RESISTANCE TO THE TICK *Boophilus microplus* ON CATTLE IN COLOMBIA: SKIN TESTING TO SELECT RESISTANT CATTLE UNDER TROPICAL CONDITIONS

Efrain Vicente BENAVIDES

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DECLARATION

I, Efrain Vicente Benavides, declare that this thesis was composed by me, and that the work described therein was my own.

DEDICATION

This thesis is dedicated to

my wife Diana Marcela,

with love, gratitude and appreciation

for her patience, tolerance and support.

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ABBREVIATIONS

AFZ	Australian Friesian Sahiwal
AIS	Australian Illawarra Shorthorn
AMZ	Australian Milking Zebu
ASP	ammonium sulphate precipitate
BOOM	Boophilus microplus
CTVM	Centre for Tropical Veterinary Medicine
DEAE	diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
g	centrifugal force
GSG	salivary gland homogenate
HPI	hours post injection
ICA	Instituto Colombiano Agropecuario
ICE	index of conversion efficiency
IgE	immunoglobulin E
IgG	immunoglobulin G
Kc/s	kilocycles per second
kD	kilodaltons
LIMV	Laboratorio de Investigaciones Médicas Veterinarias
m a.s.l.	metres above sea level
P	probability over the null hypothesis
PBS	phosphate buffered saline
psi	pounds per square inch
r	correlation coefficient (Pearson's)
r'	Spearman's correlation coefficient
RAPP	Rhipicephalus appendiculatus

SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T1	test one
T2	test two
T3	test three
T4	test four
TBS	Tris buffered saline
W	Kendall's coefficient of concordance

ABSTRACT

The feasibility of using, in Colombia, skin testing for selecting cattle of good ability to acquire resistance to the tick Boophilus microplus was investigated. The skin test used tick antigens isolated by chromatographic fractionation of whole ticks or dissection and homogenisation of salivary glands. Antigens were injected intradermally and the size of the macroscopic reactions was measured. The protein composition and antigenicity of different batches of antigens were compared by electrophoresis with polyacrylamide gels and by Western blotting. The enzyme-linked immunosorbent assay was used to relate anti-tick antibodies to individual resistance to ticks. A preliminary investigation used rabbits infested with Rhipicephalus appendiculatus ticks, then different groups of cattle in four separate experiments were infested with B. microplus and individual resistance to ticks assessed by counts of ticks.

Skin test reactions in rabbits were mainly of the delayed hypersensitivity type. Their correlation with individual resistance was positive and significant. Skin test reactions in cattle were immune specific and mainly of the immediate hypersensitivity type. Their correlation with individual resistance was highly variable between individuals and experimental groups and showed to be affected by environmental factors. The reproducibility of isolation of batches of antigens from ticks was good. Antibody titres correlated positively with low resistance to ticks, but high antibody levels interfered with the skin test reactions. It was concluded that this skin test could be used with the serological

test for the practical selection of animals that have already acquired resistance by natural infestation. The test could also be used to measure the adaptability of individuals to environmental stress.

CHAPTER ONE:

GENERAL INTRODUCTION

Colombia is an extensive country of 1,138,914 sq km with a population of around 32 million. The geography of the country is characterized by three ranges of mountains running from south to north, where most of the population is concentrated (figure 1.1). Being located in the tropical belt, Colombia is a country in which the climate is characteristic of the tropics, but the altitude is a major factor controlling the temperature. In Colombia it is therefore possible to differentiate the so called thermic steps as follows. Areas from 0 to 1000 metres above sea level (m a.s.l.) are called hot land, with a mean temperature of above 23°C; areas from 1000 to 2000 m a.s.l. are called mild land with temperatures between 18-23°C; areas from 2000 to 3000 m a.s.l. are the cold land with temperatures between 12-18°C. Areas above 3000 m a.s.l. have temperatures under 12°C where vegetation and animal life is scarce (Instituto Geografico Agustin Codazzi, 1969).

Major rivers (Cauca and Magdalena) flow between the mountains forming very fertile tropical valleys. These valleys are located under 1000 m a.s.l. and they extend to the north up to the Atlantic coast. Different ecosystems are found on the slopes of the Andean mountains, as altitude increases. High plateaux of very fertile and cold land are found above 2000 m.a.s.l. in the mountains. To the east of the country wide lowland infertile savannas are found. These savannas are sub-divided into the llanos (tropical well-drained savannas, isohyperthermic) and the Amazonas basin (tropical rain

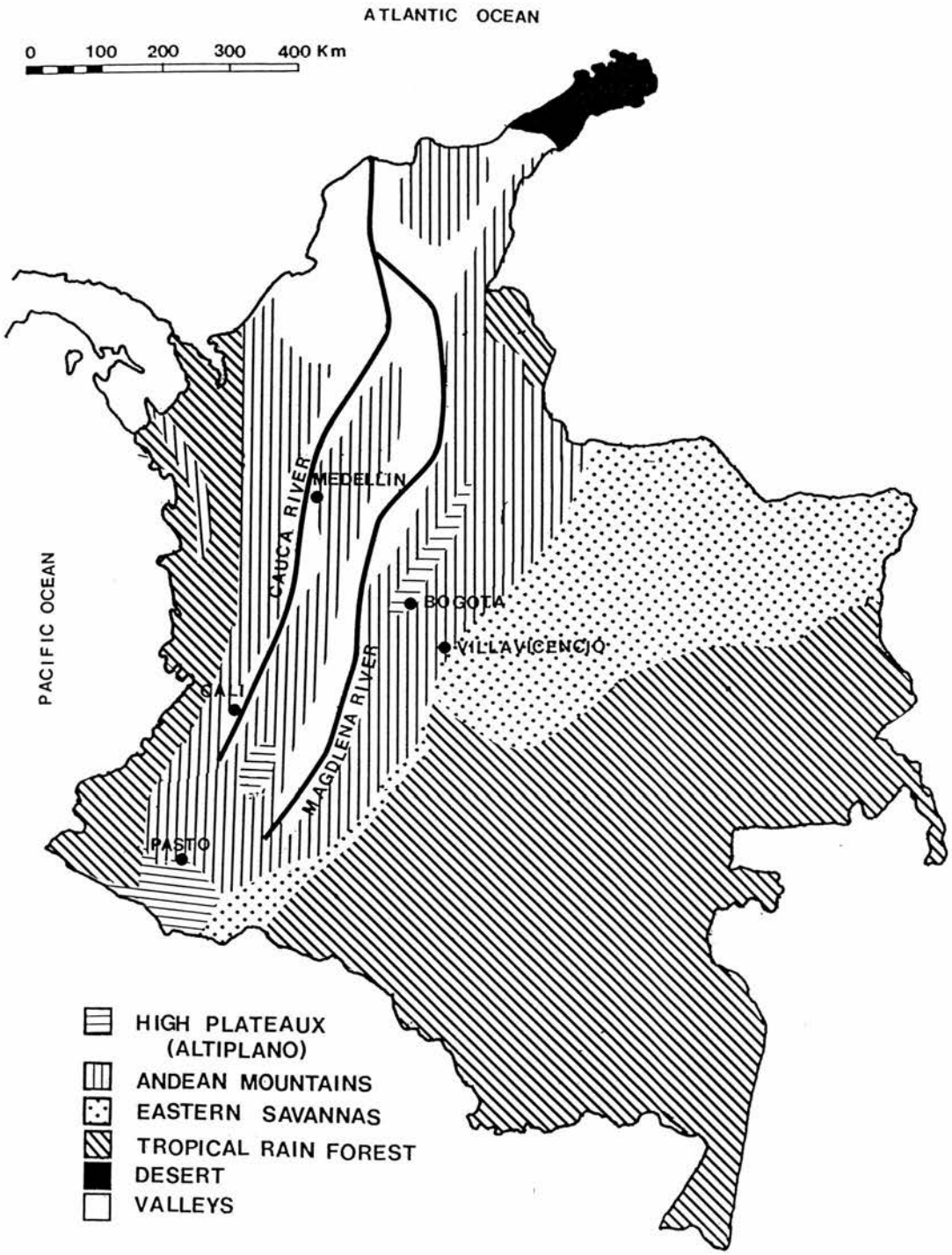


Figure 1.1.- Map of the Republic of Colombia showing the major ecological areas of the country.

forest) (CIAT, 1981).

Cattle population around 25 million head concentrates in the valleys and in two flat areas, the fertile north coast and the eastern savannas. Dairy breeds of high production but also high susceptibility to ticks and other tropical parasitic diseases, are kept on high and cold plateau where land is expensive (near Bogotá and Pasto). In recent years milk production has been increasingly moved to the valleys and the foothills of the llanos where land is less expensive and good transport infrastructure can be found.

Ticks are endemic in the country, but they only breed in areas under 1,800 m a.s.l. (figure 1.1). The tropical cattle tick Boophilus microplus, is considered as a major constraint for improved animal production in the tropical grazing areas of Colombia, where it is known to transmit Babesia bigemina, Babesia bovis, and Anaplasma marginale (Todorovic, 1976; Vizcaino, 1980). These diseases are endemic throughout the country in all the areas suitable for tick survival. Losses are associated with reduced productivity but sometimes deaths occur due to tick borne diseases, mainly when adult unprotected animals are moved from tick free area to tick infested area of the country. Problems caused by ticks have been growing recently due to the increase in the animal density because of the use of improved pastures and the introduction of more susceptible animals for dairy production in tropical areas.

Tick control in Colombia has been based on the use of acaricides, by dipping, spraying or topical application with a piece of cloth. However, these means of control are seldom used on a rational basis. Control of anaplasmosis and babesiosis has been based on the widespread use of acaricides and, to a lesser degree

the use of specific chemotherapy and vaccines, but ticks develop resistance to chemicals making control difficult and unprofitable (Dolan, 1988). Reduced efficacy of acaricides in a endemic area with heavy tick control could lead to deaths due to haemoparasitic diseases (Mahoney, 1974).

In recent years there has been increased interest in an approach to tick control based on the use of cattle which are resistant to infestation with the tick, expressed as the ability of cattle to limit the numbers of ticks that feed on them according the type of cattle (Utech, Wharton and Kerr, 1978). The degree of this resistance in the animals has traditionally been measured by artificially infesting the animals with a known number of larvae and then counting the resulting adult females that complete their cycle (Wharton, 1976). However, the fact that the animals should have a tick free period prior to the artificial infestation and the necessity to apply live ticks over the animals, makes it difficult to use these techniques extensively under field conditions. Therefore a simpler technique for use in the field would be preferable.

The relationship between hypersensitivity reactions to the inoculation of tick derived proteins and the resistance to the ticks, has led researchers to look for a skin test as a practical tool for selection of animals on the basis of their resistance (Willadsen et al., 1978), or in order to know if animals have been previously exposed to ticks (Binta and Cunningham, 1984). Such a test needs a tick-derived protein of relatively easy acquisition in large quantities. For this purpose, the separation of the proteins contained in extracts of larval ticks, using methods of

chromatographic separation has been the preferred procedure (Willadsen and Williams, 1976; Binta and Cunningham, 1984).

The general objectives of this work were as follows. To purify an antigen from the tick B. microplus to be used in skin tests developed to detect the degree of resistance to the ticks of cattle previously exposed to the tick. To compare the skin test with the standard method used to measure resistance to the tick in cattle, that is the method of artificial infestations with known numbers of larvae. To test the feasibility of application of the skin test under the conditions of the tropical areas of Colombia as a tool for the selection, by breeding and culling, of cattle herds with good resistance to the tick.

These tasks might have not been considered as an appropriate subject for a PhD thesis, if all the practical work was going to be conducted in laboratories of a university in a developed country, since it is almost 20 years ago that such a work was described in Australia. However, facilities for research in a developing country contrast with those found in a developed country where no shortage of materials, reagents or sophisticated equipment exists. Under those conditions the application of basic research is difficult even using relatively unsophisticated techniques.

Under such limitations, one of the objectives of the study was in itself, to study the problems of introduction of technology produced in developed countries into a country on its way to development like Colombia. Thus a big effort during this study was on research to adapt methods from advanced laboratories to the facilities available in Colombia. The importance of appropriate

education given to students of third world countries has also been highlighted (Thurston, 1988).

To conduct the study, the facilities available at various research centres of the Instituto Colombiano Agropecuario (ICA) were used. ICA, who sponsored this study, is an official institution devoted to agricultural research funded by the Ministry of Agriculture. It possesses many research stations in different agricultural ecosystems of the country, where research on beef and dairy cattle production is conducted. It also runs a net of veterinary diagnostic centres throughout the country, and a major central veterinary research laboratory, the Laboratorio de Investigaciones Mèdicas Veterinarias (LIMV) in the capital Bogotà.

Most of the laboratory work was conducted at LIMV. Field experiments were conducted in two research centres located on different climatic areas: La Libertad research centre located near Villavicencio at 336 m a.s.l., with a mean temperature of 26°C and an average annual rainfall of 3110 mm, and the Tibaitatà research centre located near Mosquera at 2640 m a.s.l. with a mean temperature of 16°C (figure 1.2).

The study was started in 1986. Training and preliminary experiments were conducted at the Centre for Tropical Veterinary Medicine (CTVM) of the University of Edinburgh, as an introduction to methods in chromatographic separation of proteinaceous materials and in the skin test working with a laboratory model (figure 1.3).

On arrival in Colombia, it was necessary to set up a laboratory colony of B. microplus free of haemoparasites, which provided ticks in large quantities for larval extracts and standardized vials for artificial infestations. This work was done at La Libertad and later

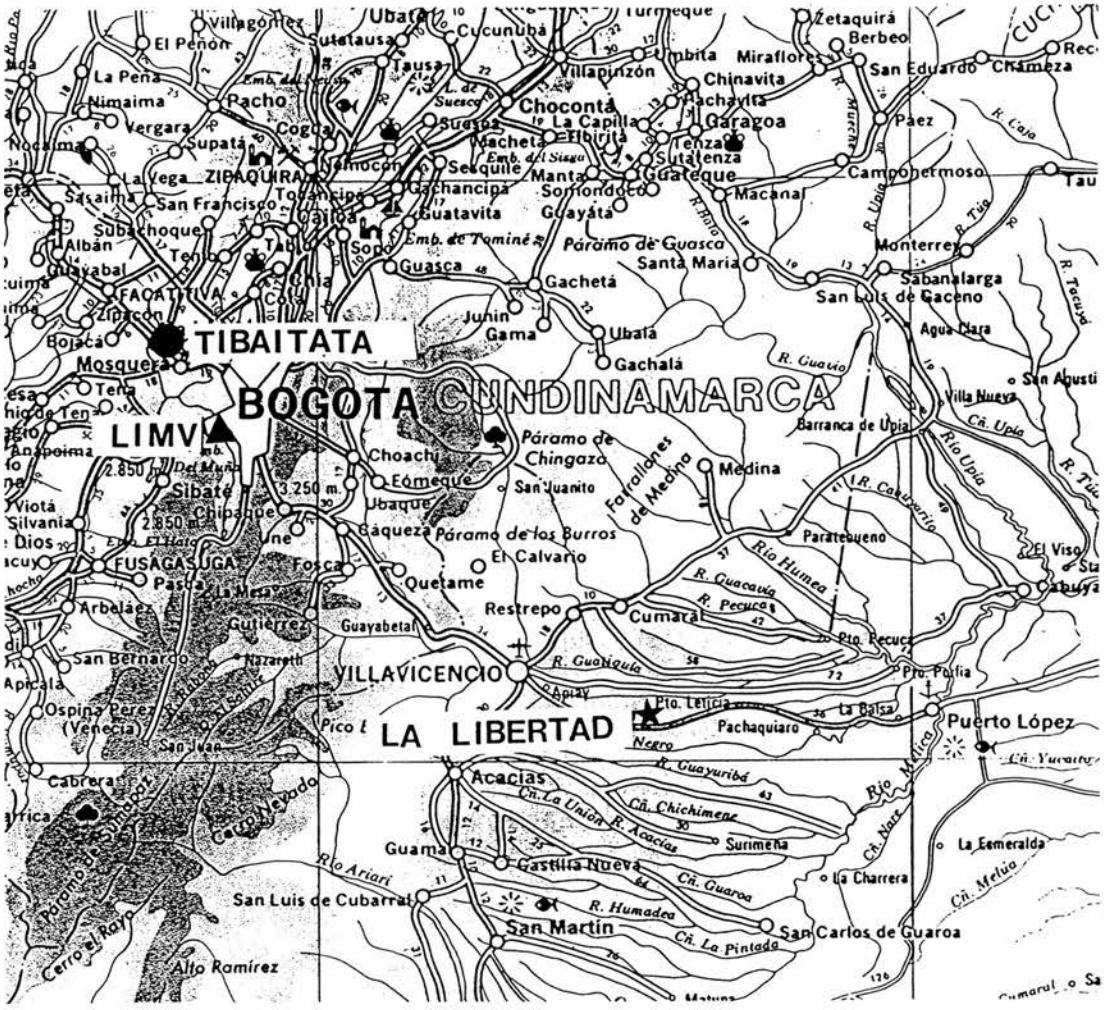
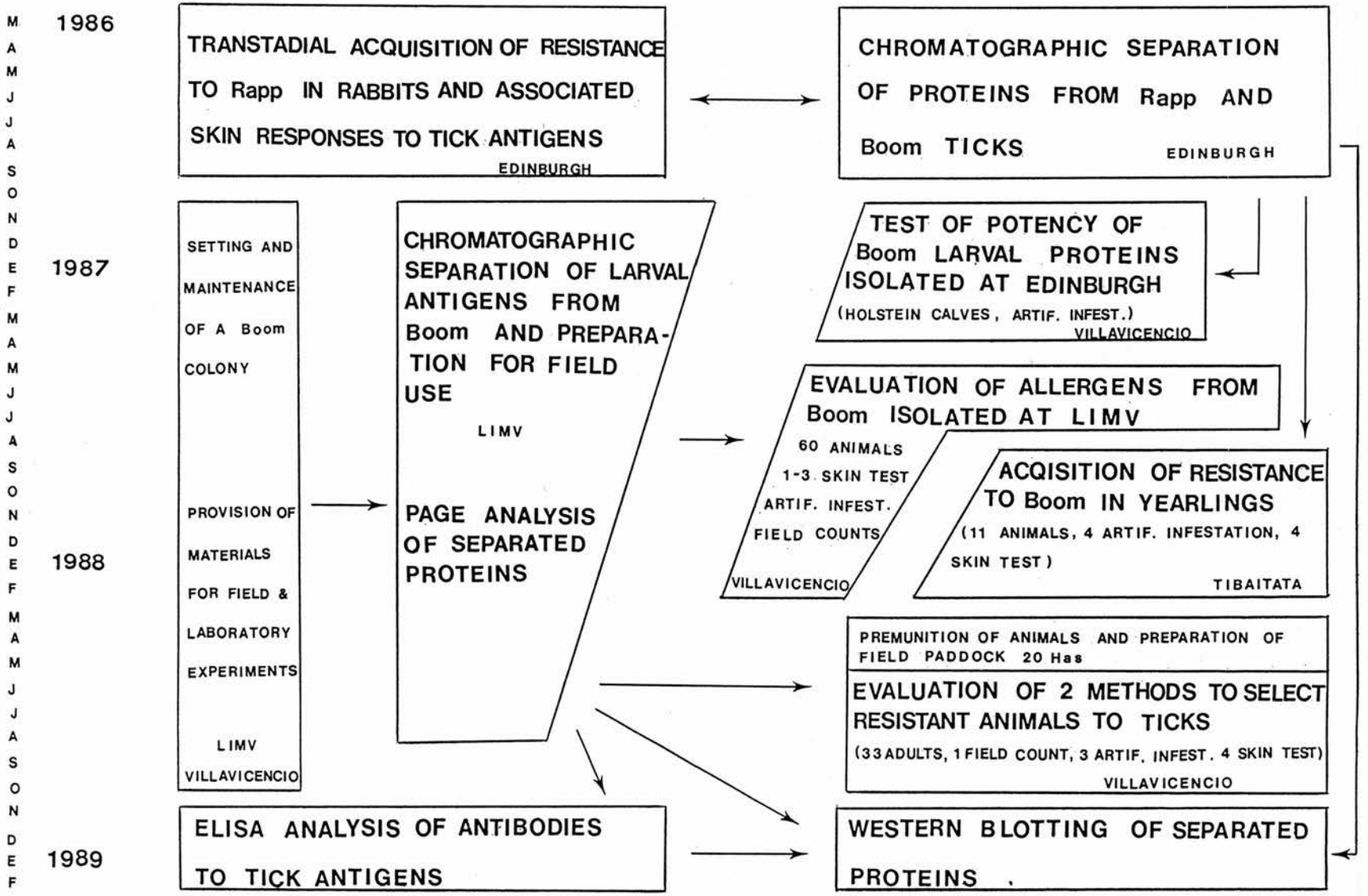


Figure 1.2.- Location of: La Libertad research centre on the western plains (Llanos) of Colombia, Tibaitatá research centre and LIMV central laboratory in the Bogotá plateau.

Figure 1.3. General chronology of the experiments conducted from March 1986 to February 1989 to study the resistance to the tick Boophilus microplus on cattle in Colombia, and the use of a skin test to select resistant animals. Experiments were conducted either at the Centre for Tropical Veterinary Medicine (CTVM) in Edinburgh, or at different locations of the Instituto Colombiano Agropecuario (ICA) in Colombia.
Rapp = Rhipicephalus appendiculatus
Boom = Boophilus microplus


M A M J J A S O N D E F M A M J J A S O N D E F M A M J J A S O N D E F
1986
1987
1988
1989
TRANSTADIAL ACQUISITION OF RESISTANCE TO Rapp IN RABBITS AND ASSOCIATED SKIN RESPONSES TO TICK ANTIGENS
EDINBURGH
CHROMATOGRAPHIC SEPARATION OF PROTEINS FROM Rapp AND Boom TICKS
EDINBURGH
SETTING AND MAINTENANCE OF A Boom COLONY
PROVISION OF MATERIALS FOR FIELD & LABORATORY EXPERIMENTS
LIMV
VILLAVICENCIO
CHROMATOGRAPHIC SEPARATION OF LARVAL ANTIGENS FROM Boom AND PREPARATION FOR FIELD USE
LIMV
PAGE ANALYSIS OF SEPARATED PROTEINS
TEST OF POTENCY OF Boom LARVAL PROTEINS ISOLATED AT EDINBURGH
(HOLSTEIN CALVES, ARTIF. INFEST.)
VILLAVICENCIO
EVALUATION OF ALLERGENS FROM Boom ISOLATED AT LIMV
60 ANIMALS
1-3 SKIN TEST
ARTIF. INFEST.
FIELD COUNTS
VILLAVICENCIO
ACQISITION OF RESISTANCE TO Boom IN YEARLINGS
(11 ANIMALS, 4 ARTIF. INFESTATION, 4 SKIN TEST)
TIBAITATA
PREMUNITION OF ANIMALS AND PREPARATION OF FIELD PADDOCK 20 Has
EVALUATION OF 2 METHODS TO SELECT RESISTANT ANIMALS TO TICKS
(33 ADULTS, 1 FIELD COUNT, 3 ARTIF. INFEST. 4 SKIN TEST)
VILLAVICENCIO
ELISA ANALYSIS OF ANTIBODIES TO TICK ANTIGENS
WESTERN BLOTTING OF SEPARATED PROTEINS .

on moved to LIMV. Two field experiments evaluated proteinaceous materials from B. microplus produced at CTVM; the first tested these antigens and the second tested the changes in reactivity associated with the acquisition of resistance in yearlings (chapters six and seven). Materials produced at LIMV were then evaluated in 60 animals at La Libertad, where a final experiment was conducted in 1988 (chapters eight and nine). In all the experiments that used cattle during this project, difficulties were faced in the acquisition and handling of experimental animals. This resource constituted a limiting factor in the design of the experiments. Groups of cattle were borrowed for different periods of time from the livestock research programmes of the same institution.

Finally on return to Edinburgh in 1988, sera and proteinaceous materials were brought to produce an ELISA test for the analysis of antibodies to tick antigens and to analyse the antigens contained in those mixtures using Western blotting. The standardization of these techniques at LIMV had been initially contemplated but the idea was abandoned because of deficiencies in reagents and equipment.

CHAPTER TWO:

LITERATURE REVIEW

2.1.- TICKS AND TICK-BORNE DISEASES.

2.1.1.- Preliminary remarks.

Ticks and tick-borne diseases are of importance to humans and livestock throughout the world (Balashov, 1972). Ticks cause direct losses through tick worry, toxicosis and hide damage and indirect losses as vectors of a number of major disease causing organisms (Dolan, 1988). Boophilus microplus is a particularly important parasite of cattle in Australia, Asia, Central and South America and Africa (Bennett and Wharton, 1968).

Economic losses due to B. microplus infestations have been estimated to be around US $7/head/year (Anonymous, 1984). This figure includes direct and indirect losses and the cost of control. For beef cattle, it has been stated that 1 kg of liveweight gain is lost per 1400 ticks infesting one animal in one year (Sutherst, Norton and Maywald, 1980), but it has been highlighted how losses in productivity are more pronounced when European breeds of cattle are raised in tropical environments (De Alba, 1977; Frisch, 1981).

Various diseases are transmitted by B. microplus, namely Babesia bovis, B. bigemina and Anaplasma marginale. However, in areas permanently infested with ticks, deaths are minimal and the infection itself does not appear to cause significant production loss, because these parasites have characteristics which allow the populations of disease organisms to reach a stable situation,

without normally causing clinical symptoms to their host. This phenomenom named enzootic stability, has been exhaustively studied for bovine babesiosis (Mahoney, 1974; Dallwitz et al., 1986), and although the transmission of anaplasmosis has different features than that of babesiosis, the concept of enzootic stability is considered also valid for this entity (Paull et al., 1980; Dallwitz et al., 1986).

Enzootic stability of babesiosis in an environment is a condition in which there is a high percentage of infected cattle but very rare occurrence of clinical disease. This relationship, that requires a high transmission rate of the parasite and consequently a relative abundance of ticks, is maintained by the infection of all calves before nine months of age. If infected then, calves will show no signs of clinical disease because of an age related innate resistance that is reinforced by passive maternal immunity (Mahoney, 1974). After this, calves acquire a solid resistance that is maintained in adult cattle by continuous reinfections with the parasite.

In regions marginal for tick survival, or where the population of ticks has been artificially reduced, this balance is not maintained, because not all calves acquire the infection with babesia before the nine months age. A proportion of susceptible adult cattle is thus created, and if exposed to infected ticks will develop clinical disease. This condition is considered as enzootic instability, in which the clinical disease is periodically apparent, coincidently with increases in the tick population due to climatic factors or to failures in tick control (Mahoney, 1974). In this way,

an intense control of ticks could change a stable situation into unstable with risk of occurrence of clinical cases of tick fever. It has been suggested that situations potentially unstable for babesiosis would develop more readily in Bos indicus herds (Mahoney, 1980).

The control of ticks and tick-borne diseases has relied on the use of acaricides, either in dips or sprays, for the last 80 years, but the development of resistance to many acaricides has created problems in this approach (Dolan, 1988). The cost of the acaricides and the infrastructure required for their application reduce profitability of cattle rearing (De Alba, 1977), and acaricides cause toxic problems with toxic residues in cattle and the environment.

Alternative measures using biological control such as pasture spelling, and the use of tick resistant cattle (Wharton et al., 1969), have been recommended to reduce the reliance in the use of acaricides. It has been demonstrated that a combination of these methods with the application of acaricides, under an integrated pest control approach, is the more economic alternative for control under the majority of situations (Norton, Sutherst and Maywald, 1983). Host resistance is seen as an essential basis for any integrated control approach. In recent years the enhancement of host resistance by immunization has received a great deal of attention and successful immunization of cattle against B. microplus has been reported (Johnston, Kemp and Pearson, 1986; Opdebeeck et al., 1988).

In Colombia, the economic impact of the haemoparasitic diseases on the productivity of herds maintained in the tick infested areas has not been determined, but reports of outbreaks of tick fever, are

usually associated with movement of animals from the tick free to the tick infested regions of the country. Economic losses due to the tick and tick-borne diseases in Colombia have been estimated to be around US $17 million annually (Cardenas, 1987).

2.1.2.- Control of ticks in Latin America.

Chemical control is the main form of tick control used in Latin America, and was initiated at the beginning of the century with arsenic compounds (Patarroyo and Costa, 1980). Eradication campaigns for B. microplus have been attempted in Argentina since 1938 (Mangold et al., 1986) and in Mexico since 1960 (Woodham et al., 1983), but a tick free situation is difficult to maintain because of the appearance of strains of ticks showing resistance to acaricides. In Brazil, resistance to organophosphorus acaricides in B. microplus ticks has been documented (Patarroyo and Costa, 1980). The Mexican eradication campaign from 1975 to 1980 cost more than US $294 million (Woodham et al., 1983).

The use of cattle resistant to infestation with the tick (2.2.1) B. microplus has been highlighted as an alternative for tick control in Latin America, with the purpose of co-existing with the tick so reducing both the costs of cattle production and reliance on use of acaricides for tick control (De Alba, 1977). In this way the Criollo cattle (Bos taurus cattle of Spanish origin and adapted to tropical environment) constitutes a genetic resource for these purposes (De Alba, 1981).

Few studies have been conducted to study the susceptibility or resistance to the tick of these native breeds. In Argentina, Mangold

et al. (1986) studied the distribution of tick counts in Hereford, Criollo and Nellore heifers. Lower tick burdens were found on the Nellore group and intermediate levels of infestation were observed on the Criollo group, but the differences were not significant in all the seasons evaluated. Acquisition of resistance to B. microplus has been described in Holstein cattle under Cuban conditions (De la Vega, Guerrero and Diaz, 1980). Under those tropical conditions they indicated that the resistance markedly decreases if a poor diet is supplied and increases on improving the nutritional status of the animal.

In Latin America and particularly in Colombia, various efforts have been made to explain the annual cycle of B. microplus under tropical and subtropical conditions. In Paraguay, highest tick counts were recorded during August to October (Quinlan, Scarone and Laneri, 1980), and the limiting factors were stated to be high temperatures during summer and water relations. In a study in a well-drained savanna in the eastern plains of Colombia (Aycardi et al., 1984), increases in the tick burdens in animals grazing Brachiaria decumbens were observed towards the middle of the rainy season (August). It was very prominent then, that two of the 16 experimental heifers carried consistently higher tick loads. The fact that a small proportion of the herd carry the majority of ticks is well recognized (Sutherst and Utech, 1981), and is a manifestation of a skewed distribution of host resistance in the population. This indicates the importance of resistant animals in controlling the whole population of the tick, this is discussed below (2.3.4).

More recent reports about the fluctuations in the populations of B. microplus ticks in various breeds of cattle in the Brazilian savanna (Gomes et al., 1989), described the occurrence of three annual peaks on tick populations, in February, May and September. In that area the dry season occurs from April to September and Nellore cattle carried significantly fewer ticks throughout the study.

Fluctuations of tick populations on a mixed herd containing European, Criollo and Zebu cattle in the eastern savannas of Colombia have been reported (Benavides, Villar and Gonzalez. 1988). In this location a marked seasonal pattern was described with a first peak occurring at the end of the dry season (February-April) and a second peak being evident at the end of the rainy season (September-December). It was suggested in this study that the nutritional stress that occurs during the dry season causes losses in the levels of resistance to the tick, which is a factor that regulates tick populations in the area of study.

2.2.- RESISTANCE TO TICKS.

2.2.1.- Definition.

Integrated control methods against B. microplus have been reported (Norton, Sutherst and Maywald, 1983). These methods include three major options for improved tick control: to improve the chemical control, to reduce the host-finding rate and to increase host resistance. This last approach to tick control is based on the use of cattle which are resistant to infestation with the tick B. microplus (Wharton, Utech and Turner, 1970).

Resistance in wide terms can be defined as the capacity of the

host to impose limitations upon the parasite at any stage of its relationship with the host (Wakelin, 1978). These attributes can be both innate and acquired. On the other hand, susceptibility implies that the host provides an appropriate environment for the establishment, development and maturation of the parasite. These two concepts will be referred to in the context of this work as antonyms.

In the case of B. microplus ticks and cattle, resistance has been referred to as the ability of cattle to limit the number of ticks that survive to maturity (Utech, Wharton and Kerr, 1978). This resistance generally has been associated with immunity, requiring previous exposure to the parasites in order to be expressed (Wagland, 1978a). Alternatively, the occurrence of innate resistance has been claimed. Factors related with this resistance include the thickness and the structure of the hide (Bonsma, 1981; Wagland 1978b) and the follicle depth (Wilkinson, 1962).

2.2.2.- Mechanisms of resistance to B. microplus in cattle.

The main expression of resistance to B. microplus in Bos taurus (European cattle) is the rejection of larvae at the time of attachment (Roberts, 1968), but resistance is also expresed against other instars during moulting and reattachment. The weights at engorgement of female ticks are reduced and the length of feeding is increased on animals displaying resistance (Wagland, 1975).

The production of strong cutaneous reactions at tick attachment sites on resistant animals, were described by Riek, (1962). Those reactions were characterized by intense infiltration of the dermis

by eosinophils, other polymorphonuclear cells and lymphocytes forming distinct pustules around the mouthparts of the tick. In addition, Schleger et al. (1976) described eosinophil accumulation and mast cell degranulation at attachment sites in highly resistant animals. These reactions were less pronounced in animals of low resistance and almost absent in unexposed animals. These findings lead to the conclusion that an immediate hypersensitivity reaction was important in the rejection reaction (Wikel and Allen, 1982). All the immunological terms are as defined by Roitt, Brostoff and Male (1985).

It has been suggested that this hypersensitivity reaction produces an increase in the grooming activity in resistant animals, which causes a decrease in the tick burden (Hewetson and Nolan, 1968). In addition, histamine itself has a direct effect on the tick attachment behaviour (Kemp and Bourne, 1980).

The hypersensitivity reactions are directed to components of the saliva of the tick. Geczy et al. (1971) described immediate inflammatory reactions to the injection of whole saliva on tick exposed cattle, and indicated that saliva contains a macromolecule with esterase activity. Schleger and Lincoln (1976) described the deposition in the dermis of an esterase, 4 hours after the attachment of larvae on susceptible cattle. On the other hand, various proteinaceous components with allergenic activity in animals exposed to the tick have been separated from unfed ticks and some have been highly purified (Willadsen and Williams 1976; Willadsen et al., 1978; Willadsen and Riding, 1979).

When the so called Allergen 1 and Allergen 2 were tested in

cattle with differing degrees of resistance, the sensitivity to the allergens were correlated with the level of resistance (Willadsen et al., 1978). These reactions were shown to be immunoglobulin E (IgE) specific as demonstrated by their capacity to be transmitted passively by sera, producing Prausnitz-Kustner reactions on recipient calves.

Other factors influencing these hypersensitivity reactions have been studied (Willadsen, 1980b). The total amount of histamine in the skin and serum levels of antibodies to one allergen, correlated with resistance, suggesting that a major factor influencing the hypersensitivity reactions is the amount of mast cell bound specific IgE. Furthermore Hales et al. (1981) observed greatly increased skin capillary blood flow in cattle exposed to B. microplus larvae and the degree of hyperaemia was directly related to the level of tick resistance.

Production of antibodies to the infestation with B. microplus have been described. Brossard (1976) reported the presence of antibody responses against B. microplus salivary antigens, that were detected using the indirect immunofluorescent technique. He also demonstrated that the immune bovine sera contains two precipitating systems to those antigens, and that rabbits immunized with salivary glands produced up to seven precipitating systems. He finally indicated that the presence of antibodies was associated with protection (resistance). On the other hand, the production of antibodies directed against phosphomonoesterases of B. microplus larvae in cattle after exposure to the tick has been described (Reich and Zorzopulos, 1980).

2.2.3.- Mechanisms of resistance to other species of ticks.

The immunological basis of host resistance in laboratory animals has been widely studied. It is currently believed that antibody, cell mediated and complement-dependent immune effector mechanisms are active in the expression of acquired resistance to tick infestation (Wikel and Whelen, 1986). The role of vasoactive amines in host rejection of ticks, has also been highlighted (Wikel, 1982). A delayed cell mediated reactivity appears to be a more important mechanism for the expression of resistance. Gregson (1970) demonstrated in himself a hypersensitivity reaction and response of sensitized lymphocytes to cement and saliva of the tick Dermacentor andersoni. Cell mediated immune components have been described in the resistance to D. andersoni in guinea pigs (Wikel, Graham and Allen, 1978). They demonstrated that peak lymphocyte in vitro responsiveness to salivary antigens occurred 24 hours after the initiation of a second larval infestation. This responsiveness was associated with a large number of basophils being attracted to the tick attachment site.

Accumulations of basophils have been observed at attachment sites of many tick species on different hosts including: Amblyomma americanum in guinea pigs, (Brown and Knapp, 1980), Rhipicephalus appendiculatus in guinea pigs, (Mc Laren, Worms and Askenase, 1983), Hyalomma anatolicum anatolicum in rabbits, (Gill and Walker, 1985), and A. americanum in cattle, (Brown, Barker and Askenase, 1984). On the other hand, histological changes in sensitized hosts have been described for R. appendiculatus in cattle, (Fivaz, Norval and Brown, 1984; Walker and Fletcher, 1986). These changes were

characterized for epidermal vesiculation and infiltration of the dermis by eosinophils and neutrophils.

A similar pattern of changes was observed for H. anatolicum anatolicum in cattle, (Gill, 1986), but he described that the number of basophils increased as the feeding advanced. Conversely for B. microplus in immune cattle eosinophils were the primary cells found at tick attachment sites (Amin-Babjee and Riek, 1986), but neutrophils and basophils demonstrated an increase in numbers after the sixth repeated infestation. They also described oedematous epidermal lesions that were vacated by larvae. In those lesions neutrophils were predominant, but eosinophils and basophils were found in substantial numbers. This indicates that the immune reactions observed on different tick-host relationships bear common immediate and delayed rejection mechanisms but that they are expressed with different intensities in each interaction.

Finally, it has been suggested that tick infestation impairs the host immune competence in spite of the expression of resistance (Wikel and Whelen, 1986). They hypothesised that this impaired host immune competence could facilitate tick feeding and transmission of vector borne pathogens. However, Callow and Stewart (1980) did not observe increased frequency of parasitaemia with B. bovis in cattle parasitized with B. microplus, but described a reciprocal effect of babesiosis on resistance to the tick.

2.3.- TICK CONTROL USING RESISTANT ANIMALS.

2.3.1.- Measurement of resistance.

The resistance of cattle to the tick that has been acquired after repeated exposure is generally measured by counting the number of female ticks 4.5 to 8.0 mm in length (standard ticks) on one or both sides of the animal (Wharton and Utech, 1970). Ticks of this size will detach and drop from the cattle during the following day. This method can be used to estimate field populations of ticks, to rank a group of cattle in relation to their tick burdens. This procedure is termed ranking and requires standardized conditions for the animals being compared to take account of the host and environmental conditions affecting resistance (Sutherst and Utech, 1981). The accuracy of this method has proved acceptable under medium and high stocking rates, but the repeatibility of these rankings under field conditions of low stocking rates, requires investigation (Sutherst and Utech, 1981).

Under experimental or stall conditions, the assessment of resistance is made by calculating the yield of mature female ticks as a ratio of a number of larvae artificially applied, which generally is 20,000 larvae (the equivalent to 1 g of eggs). Female ticks produced are counted on days 18 to 22 after the cattle were infested on day 0 (Utech, Seifert and Wharton, 1978). This measurement of resistance is known as rating. Approximate ratings can be made by reducing the number of days on which ticks are counted and this is sufficiently accurate for most commercial conditions (Sutherst and Utech, 1981).

The artificial infestation method has the limitation of requiring a tick free period before ticks are applied to the cattle (Utech, Seifert and Wharton, 1978), but it has been demonstrated under practical selection programmes, that the use of tick free

pastures is not essential, and that the counting can be restricted to one or two days following one infestation (Seifert, 1984).

2.3.2.- Factors affecting tick numbers in the field.

In general terms, the study of the fluctuations that occur in each of the different stages of the life cycle of the tick B. microplus, can be divided into three components: a. Developmental phases in vegetation (non-parasitic), b. Host finding process, and c. The parasitic phase (Sutherst, Wharton and Utech, 1978).

For the non-parasitic phases, briefly, the major mortality factors known for ticks are extremes of temperature and desiccation (Sutherst, Wharton and Utech, 1978). On the other hand, all the population processes which include the fecundity of detached female ticks, the egg development and survival and the larval survival are mainly affected by the temperature and the moisture provided by the microclimate created for any particular pasture in an environment (Harley, 1966). That microclimate is affected by the different seasons that occur at different locations (Norton, Sutherst and Maywald, 1983).

The host-finding process is mainly affected by the cattle density and movement. The host density is regulated by the quantity and quality of the pasture, which is affected by the weather and also by managerial activities like pasture spelling or rotation used to control ticks. The movement or host grazing behaviour is affected by the cattle breed and by the host density and the weather (Sutherst, Wharton and Utech, 1978).

2.3.3.- Effects of the use of resistant animals on tick populations.

A multiplicity of factors are known to be responsible for the fluctuations in numbers of ticks that complete their cycle on cattle (Sutherst, Wharton and Utech, 1978). Of these, there are factors related to the animal, to the quantity and quality of the pasture and to the season.

One of the major factors affecting the parasitic phase of the cattle tick is the susceptibility of the host. The susceptibility of a host is given by the proportion of attaching ticks which feed successfully on it and is the converse of host resistance (Sutherst, Wharton & Utech, 1978). This susceptibility can be directly measured using methods of artificial infestation and varies from 0 on unfavourable species up to values of 0.20 in some breeds of European type cattle (Sutherst and Utech, 1981).

Long term population studies of B. microplus on cattle of different levels of resistance have been conducted in Australia, using untreated animals (Sutherst et al., 1979). Lower tick counts were observed on a herd selected for resistance to the tick but important fluctuations were observed according the season, the year and the reproductive status of the animals. That indicated that the level of susceptibility to the tick partly determines the equilibrium population sizes of ticks in a given environment.

The effect on the whole population of the tick B. microplus of the use of resistant animals as an alternative for tick control has been studied using a modelling approach (Sutherst, Norton and Maywald, 1980; Norton, Sutherst and Maywald, 1983). Under the

conditions prevalent in Southern Queensland, taking into account the seasonal variations in tick yield, the effect of specific levels of resistance on tick numbers was assessed. The value of resistant animals in controlling tick numbers was more marked in the autumn. It was demonstrated that as resistance declines, tick numbers and losses in liveweight increase exponentially (Sutherst, Norton and Maywald, 1980).

Host resistance has been claimed to be an essential basis for any integrated control approach for ticks. Selective breeding, culling animals with low tick resistance and supplementary feeding, have been discussed as possible ways to increase host resistance in animal populations. (Norton, Sutherst and Maywald, 1983). The change to Zebu-cross cattle is seen as a prerequisite to long-term improvement in tick control (Sutherst and Utech, 1981).

2.3.4.- Factors affecting resistance.

The first and most known factor affecting resistance to the tick is the exposure to a sufficient tick challenge. After this, cattle acquire a characteristic general level of resistance that is associated with the breed type of the animals (Hewetson and Nolan, 1968; Wagland, 1975; Utech, Wharton and Kerr, 1978).

It is well known that cattle of British breeds (Bos taurus) have a greater susceptibility to the tick than do Zebu breeds (Bos indicus) (Riek, 1962), but individuals with high resistance occur in all breeds (Wharton et al., 1969). In a study conducted in Queensland (Utech, Wharton and Kerr, 1978), animals were ranked based on the estimation of the percentages of infesting larvae which

failed to reach maturity. Cattle were classified into those of high (>98%), moderate (95-98%), low (90-95%) and very low resistance (<90%). The proportion of highly resistant cattle in the various dairy breeds were: Jersey, 70%; Australian Illawarra Shorthorn (AIS), < 10% and Friesians, < 10%. In the beef breeds the proportions of highly resistant cattle were: Brahman x AIS, 65%; Droughtmaster, 52%; Santa Gertrudis, 36%; Bradford 31%; Hereford x Shorthorn (HS), 3%; Shorthorn, 0% and Hereford, 0%.

Many other factors are known to reduce temporarily the resistance of cattle to ticks. These factors can be divided into those related to the effect of the environment (mainly nutrition), and those related to the physiology of the host (Bennett and Wharton, 1968). Changes in the level of resistance according to the time of the year have been well documented under Australian conditions. To start, Bennett and Wharton (1968), described differences in the tick yield of stalled animals, with higher yields of ticks in summer than in winter. Later, Wharton, Utech and Turner (1970), described the specific effect of the season on the expression of resistance and stated that the discrimination between animals on the basis of resistance was more reliable in summer than in winter.

Some of these seasonal effects have been attributed to nutrition. The breakdown of the resistance to the tick due to malnutrition and the consequent loss of body weight has been described (O'Kelly and Seifert, 1969), which was also suggested by Wharton, Utech and Turner (1970) as a component of the seasonal fluctuation of resistance. That finding was later confirmed by

O'Kelly and Seifert (1970), who indicated that the percentage yield of mature ticks was about three times greater in animals on low-quality than on high-quality feed.

More recently, Sutherst et al. (1983b) demonstrated that resistance varies with season, at least under Australian conditions, not only as a result of changes in the nutritional composition of the pasture, but also as a host physiological response to shortening photoperiod. They demonstrated that up to five times as many ticks survived in autumn-winter (March-July) on animals grazing native pastures as in spring-summer. But on animals nutritionally supplemented, the differences in tick yield on both seasons was 2-3 times. They demonstrated a spontaneous recovery of resistance by July and indicated that the nutritional stress accentuates the loss of resistance and delays its recovery.

Changes in the level of resistance, related to the physiology of the host are mainly those related to pregnancy and lactation, sex and age (Sutherst and Utech, 1981), but their specific effect appears to be regulated by nutrition and environmentally regulated factors. Seifert (1971) described higher tick burdens on males than on females, and on European cattle described up to 6.8 times higher tick burdens on lactating than on dry cows, but such an effect was not evident in Zebu cross cows. At the same time, Johnston and Haydock (1971) reported no effect of lactation or pregnancy on European and Zebu crossbred cattle in the tropics.

In more recent studies it has been demonstrated that pregnant cows were significantly less resistant than non-pregnant cows, and that lactating cows were very much less resistant than non-lactating cows (Utech, Seifert and Wharton, 1978). In long-term population

studies it has been shown (Sutherst et al., 1979), that the resistance of the herds, measured by artificial infestations with larvae, increased during the first 3 years, but declined after the cattle began breeding and fluctuated from year to year.

Finally, it has been recommended that when attempting selection of resistant cattle, the animals on which selections are to be made should have had similar management to reduce environmental effects (Seifert, 1984). He also stated that selection within different classes of stock, such as lactating and non-lactating cows, is a practical method of accounting for the fixed effects related with the physiological status of the animal.

2.4.- GENETIC ASPECTS OF RESISTANCE.

2.4.1.- Heritability.

The inheritance of resistance to the tick B. microplus has been widely studied and many attempts have been made to relate it to various genetic characteristics of cattle. It is now widely accepted that a wide range of resistance occurs in all breeds of cattle. The resistance is associated primarily with Zebu cattle and their crosses with European breeds (Wharton, 1976), but resistance is also present in some European cattle individuals. What follows is a chronological summary of the reported findings on both breed groups.

Hewetson (1968), studied the inheritance of resistance to experimental infestations in 59 quarter-breed Zebu steers, and described a heritability of 28% at the fourth infestation, which increased to 42% at the fifth infestation. Later, heritability based

on single tick counts, was assessed in a herd of AIS cattle (Wharton, Utech and Turner, 1970). These estimates were of 39% from dam-calf correlations and 49% from full-sib correlations, and the estimates based on summer counts were 42 and 64% respectively. They stated that the heritability was high enough to provide strong encouragement for selecting for tick resistance.

Seifert (1971) studied the tick burdens from field infestations on crossbred Zebu x European cattle. He described that the estimates of heritability in the European cattle were non significant and inconclusive. In the Zebu crossbreds, he described that there was little heritable variation in F1 cattle, but that in subsequent generations the heritability was estimated to be 82%, suggesting that the resistance in pure Zebus was dominant.

More recently, Utech, Wharton and Kerr (1978) indicated that the selection of resistant cattle in European x Zebu breeds could be achieved by culling the 20% of cattle which are below the 95% level of resistance. They stated that resistance levels below 95% were unsatisfactory for tick control using resistant cattle. Contemporarily, the tick burdens on untreated cattle selected for different levels of tick resistance were studied (Sutherst et al., 1979). They showed that the tick counts on the calves up to weaning were not correlated with concurrent counts on their dams, suggesting that their long term resistance had not been established up to weaning.

2.4.2.- Creation of tick resistant breeds of cattle.

Based on the above described inheritances of the resistance to

the tick, the breeding for resistance in a herd of European (AIS) and European x Zebu (Brahman x AIS) was carried out (Utech and Wharton, 1982). In the AIS herd, using initially strong culling rates (50%), they demonstrated that the selection and breeding of the cows and bulls resulted in genetic improvement in the resistance of the progeny. However, this procedure required 15 years (3 to 4 generations) to produce offspring of similar resistance to Bos indicus x Bos taurus crossbred cattle. Milk production tests on heifers in the AIS herds, indicated that selection for tick resistance did not select against milk production. In the crossbred herd, a high degree of resistance was obtained by the selection and breeding for tick resistance. Only one generation was required to produce the level of resistance achieved in the AIS herd.

Using these same principles, tick resistant dairy breeds have been developed in Australia. The Australian Milking Zebu (AMZ) (Hayman, 1974), and the Australian Friesian Sahiwal (AFS), (Alexander, Reason and Clark, 1984; Alexander et al., 1984). In both experiences different cross-breeding levels of Zebu and European cattle were undertaken, using Sahiwal bulls and Jersey cows for the AMZ or Friesian cows for the AFS.

In general, in both selection programmes, bulls from the F1 were bred with either European or Crossbred European x Zebu cows selected for high milk production. The selection of sires used a test for resistance to the tick and a progeny test based on the milk production of their daughters. For the AMZ they were additionally tested for heat tolerance. In the case of AFS, the animals should have a minimum of 98% resistance to be allowed for the progeny test.

2.4.3.- Negative aspects of selection for resistance and genetic markers.

Today, it is widely accepted that the capacity of an animal to display immunity and/or resistance to a pathogen is affected by changing environmental stimuli (Kelley, 1985). On the other hand, it is also accepted that the immune response while subjected to environmental influence, is under genetic control. Based on this, the selection of animals for improved immune response has been claimed as beneficial. However, these traits have been generally ignored by animal breeders (Wakelin, 1978; Warner, Meeker and Rothschild, 1987).

The effect of various environmental factors on the resistance to B. microplus on cattle has already been described. That knowledge has lead to recommendations to avoid the selection of animals for resistance to the tick at the times of the year when the general condition of all the animals is lowered due to environmental factors (Wharton, 1976; Holroyd and Stear, 1984). The selection of cattle for maximum liveweight gain in the presence of ticks has been suggested (Sutherst and Utech, 1981) as a useful procedure under the above described conditions.

Frisch (1981), however has warned that due to the interactions between level of resistance and plane of nutrition and sex, the continued selection for tick resistance, may have the undesirable effects of lowering both inherent growth rate and inherent fertility. Under stressful conditions the animals depress their feed intake (Frisch and Vercoe, 1978), and in many tropical areas the plane of nutrition is restricted for a large part of the year.

The selection for tick resistance, either directly or indirectly, by selecting for growth rate in the presence of ticks should favour animals of low growth potential, if they are subjected to the above described conditions (Frisch, 1981). In other words, it will be conducive to the selection of animals with a lower capacity to be affected by a stressful environment. This could have the undesirable consequence of limiting the capacity of an animal to respond to future improvements in the plane of nutrition. He suggested that selection should be directed towards factors of resistance which are stable under environmental conditions rather than at the expression of this resistance.

On the other hand, it has been suggested that the selection for genetic disease resistance will require identification of specific resistance genes or the identification of genetic markers linked to resistance (Warner, Meeker and Rothschild, 1987). These markers for selection of tick resistant cattle have been studied using different approaches.

Francis and Ashton (1967), looking for a possible gene marker in the selection of cattle for tick resistance, studied a group of 51 Droughtmaster and 12 Bos taurus cattle. They described a significant association between the distribution of amylase genes and tick burden in the Droughtmaster cattle, and a similar but not significant association in the Bos taurus cattle. It was suggested then that selection of animals carrying amylase type B would be expected to produce an overall decrease in tick burden, but no further reports were made on this aspect.

Various characteristics of the skin have been claimed as useful

tools for selecting tick-repellent cattle (Bonsma, 1981). These could be related to the finding of more arteriovenous anastomoses in the skin of animals with high resistance to B. microplus (Schleger, Lincoln and Bourne, 1981). No clear conclusions have been reported for the selection of animals using these characteristics.

The immediate hypersensitivity reactions to the dermal inoculation of proteins purified from larval extracts of B. microplus ticks and the levels of specific agglutinating antibodies have been correlated to the level of tick resistance in cattle (Willadsen et al., 1978), but these findings have not been used for selection purposes, apparently because these methods were developed only to study the immune mechanisms of tick resistance in cattle, and also because the association between skin hypersensitivity and resistance to the tick was far from complete.

In recent years the relationship between tick resistance and the major histocompatibility system (Roitt, Brostoff and Male, 1985; Warner, Meeker and Rothschild, 1987) has been studied (Stear et al., 1984; Holroyd and Stear, 1984; Stear et al., 1989). Varying results have been reported from calf groups analysed yearly, but one or two antigens show encouraging relationships with either resistance or susceptibility to the tick. It is interesting to note that the only month in which significant associations between major histocompatibility type and tick resistance have been found, is July, when the mean and variances of tick numbers are lowest (Stear et al., 1989).

It can be concluded that, although there are some major histocompatibility antigens showing associations with tick resistance, more work in this field is required improve in the

understanding of the genetic basis of resistance. Finally, it has been stated that the activity of the class II major histocompatibility antigens (Ir genes) could be indirectly detected by measuring antibody titres, plaque-forming cell responses and delayed hypersensitivity reactions of the classical and cutaneous basophil types (Wakelin, 1978). This encourages the use of skin tests for the selection of animals for resistance to parasites.

2.5.- SKIN TEST TO SELECT ANIMALS RESISTANT TO THE TICK.

2.5.1.- History and principles of the skin test.

Skin tests have been used for many years for different purposes. These have included: the aetiological diagnosis of disease, for example in bovine tuberculosis and human schistosomiasis; the immunological classification of individuals, for example into atopic and non atopic-groups; and the test of the immunological status of a patient, for example in immunodeficiency diseases (Pepys, 1975; El Raziky et al., 1981; Radunz and Lepper, 1985).

Skin tests are based on hypersensitivity reactions. The term hypersensitivity has been usually applied to an adaptive immune response, which occurs in an exaggerated or inappropriate form, causing tissue damage (Roitt, Brostoff and Male, 1985). Hypersensitivity is an individual characteristic and is manifested on second or subsequent contact with a particular antigen.

Skin test reactions can be caused by immediate (type I), Arthus (type III) and delayed (type IV) hypersensitivity reactions, which

have characteristic features. The first two types are antibody mediated and the last one is mediated by T cells and macrophages. The different types of hypersensitivity do not necessarily occur in isolation from each other (Roitt, Brostoff and Male, 1985). It has been stated (Pepys, 1975), that a full assessment of skin test requires readings after an interval of 10-15 minutes, 5-6 hours and 24-48 hours, and also in some cases a further reading after several weeks for the presence of granulomatous reactions.

Type I hypersensitivity occurs as a result of the release of pharmacological mediators, such as histamine, by IgE-sensitized mast cells. Immediate reactions develop within minutes, are maximal after 10-20 minutes and resolve within about 1 to 1 1/2 hours (Pepys, 1975), but a late phase that may last 24 hours has been described (Roitt, Brostoff and Male, 1985). A remarkable characteristic of this reaction is its capacity to be passively transferred to non-sensitized individuals by sera of sensitized animals, the fact on which the passive cutaneous anaphylaxis or Prausnitz-Kustner test is based. Skin tests designed to study type I hypersensitivity, have been mainly used to study allergic conditions both in humans (Pepys, 1975) and in animals (Black, 1979). However, diagnosis of parasitic infections (hydatid cyst and visceral larva migrans) has been attempted based on immediate hypersensitivity reactions to antigens of respective parasites (Pepys, 1975).

Arthus (type III) hypersensitivity, is caused by the deposition of immune complexes and the activation of the complement cascade. Reactions develop several hours after skin testing, being maximal at about 7 to 8 hours and resolving within 24 to 36 hours (Pepys, 1975). The reaction is characterized by infiltration of neutrophils

and the intravascular clumping of platelets (Roitt, Brostoff and Male, 1985). Complement activation is essential for this reaction to develop, but Arthus reactivity can not be determined on gross appearance alone (El Raziky et al., 1981).

Delayed (type IV) hypersensitivity reactions are those reactions that cannot be transferred from one animal to another by serum but can be transferred by T lymphocytes. Although the classical example of type IV hypersensitivity is the tuberculin reaction (Pepys, 1975), nowadays four types of delayed hypersensitivity reaction are recognized. The first three of these, the Jones-Mote reaction, contact hypersensitivity and tuberculin type hypersensitivity, occur within 72 hours of antigen challenge. The fourth type, granulomatous reactions develop over a period of weeks (Roitt, Brostoff and Male, 1985).

The Jones-Mote reaction (cutaneous basophil hypersensitivity) is maximal 24 hours after antigen challenge and is characterized by infiltration of basophils, accompanied by lymphocytes and mononuclear cells. Both contact and tuberculin type hypersensitivity reactions are maximal at 48 hours and are characterized by infiltration of mononuclear cells, but the first is predominantly an epidermal reaction and the second a dermal reaction. The progression from tuberculin-like to granulomatous reaction appears to depend on the persistence of the antigen in the tissues (Roitt, Brostoff and Male, 1985).

Skin tests based on delayed hypersensitivity have been used for the diagnosis of many diseases including bacterial, viral, rickettsial and fungal agents (Pepys, 1975), but the most

extensively used in cattle has been the tuberculin test in Mycobacterium bovis eradication programmes (Radunz and Lepper, 1985). In this test cattle are inoculated either in the caudal fold or in the neck and the increases in skin thickness are measured 72 hours after injection.

2.5.2.- Hypersensitivity responses and tick infestation.

The topic has already been discussed for B. microplus and cattle (2.2.2). There is considerable evidence to indicate that the reactions of immediate hypersensitivity are responsible for some of the immunity to B. microplus, but it is unlikely to be the whole cause (Willadsen, 1980a). Immediate and delayed hypersensitivity reactions have been described in the literature as a manifestation of immunity to ticks in various laboratory models.

Immediate responses to the inoculation of salivary antigens in immune guinea pigs have been described for the tick A. americanum (Brown and Askenase, 1986). Type I reactions within 100 minutes of injection have also been described for cattle and R. appendiculatus ticks (Fivaz, Norval and Brown, 1984). Using guinea pigs and D. andersoni ticks, a typical cutaneous basophil hypersensitivity was described to the dermal inoculation of salivary antigens (Wikel, Graham and Allen, 1978). Calves exposed to the same tick gave immediate and delayed reactions when skin tested with a salivary antigen (Wikel and Osburn, 1982).

Immediate (30 minutes and 5 hours) and delayed (24 hours) skin reactions to A. americanum antigens were recorded in calves exposed to the tick (George, Osburn and Wikel, 1985), and finally delayed

reactions (classical tuberculin) were described in rabbits immune to Ixodes ricinus, challenged with salivary antigens of that tick (Girardin and Brossard, 1985).

Most of the studies reviewed by now, have used the skin test to study the mechanisms of the immune responses to the tick, but few attempts have been made to use the skin test to discriminate between immune or non-immune animals for selection purposes. Willadsen et al. (1978) working with B. microplus and cattle, skin tested various herds of cattle, mainly to study the mechanisms of resistance. Using the same principles, Binta and Cunningham (1984), assayed the use of a skin test to identify cattle previously exposed to R. appendiculatus ticks, and suggested that a quantitation of this test could be used to measure the tick resistance status of cattle.

Very recently, reports have been made on the use of skin tests to assess the status of tick resistance in cattle, using R. appendiculatus salivary antigens (Smith et al., 1989; Walker and Fletcher, 1989). In field trials, Smith et al. (1989), described significant negative correlations between the intensity of the reactions and the total number of the ticks Amblyomma variegatum, R. appendiculatus, Hyalomma truncatum, Boophilus decoloratus and Rhipicephalus spp. on the animals, based on the readings of delayed hypersensitivity reactions. On the other hand, Walker and Fletcher (1989), at laboratory level described correlation of the reactions at 24 hours with resistance. The similarities and discrepancies of these results with those obtained during this study will be raised in the general discussion.

CHAPTER THREE:

GENERAL MATERIALS AND METHODS

3.1.- LABORATORY COLONY OF THE TICK *Boophilus microplus*.

3.1.1.- *Basic Colony*.

The colony of *Boophilus microplus* maintained in the laboratory of La Libertad research centre (Benavides *et al.*, 1988) was used as the source of ticks for the different experiments conducted in Colombia. The ticks originated from Zebu animals at the slaughter house of Villavicencio in 1982 and had been maintained for at least 15 generations by feeding on Holstein calves infected with *Anaplasma marginale*, *Babesia bovis* and *Babesia bigemina*, using techniques similar to those described by Sutherst, Wharton and Utech (1978). This infection with haemoparasites was not a deliberate procedure, the host calves were raised in tick infested pastures and carried subclinical infections.

The calves used for the maintenance of the parasitic stages of the colony were individually kept in a cement floored stall (3x2 m), that was enclosed by a fine mesh to prevent the entrance of flies. No protective measure was used to prevent the detached ticks being groomed off by the calves, but numbers produced were usually sufficient to ignore this.

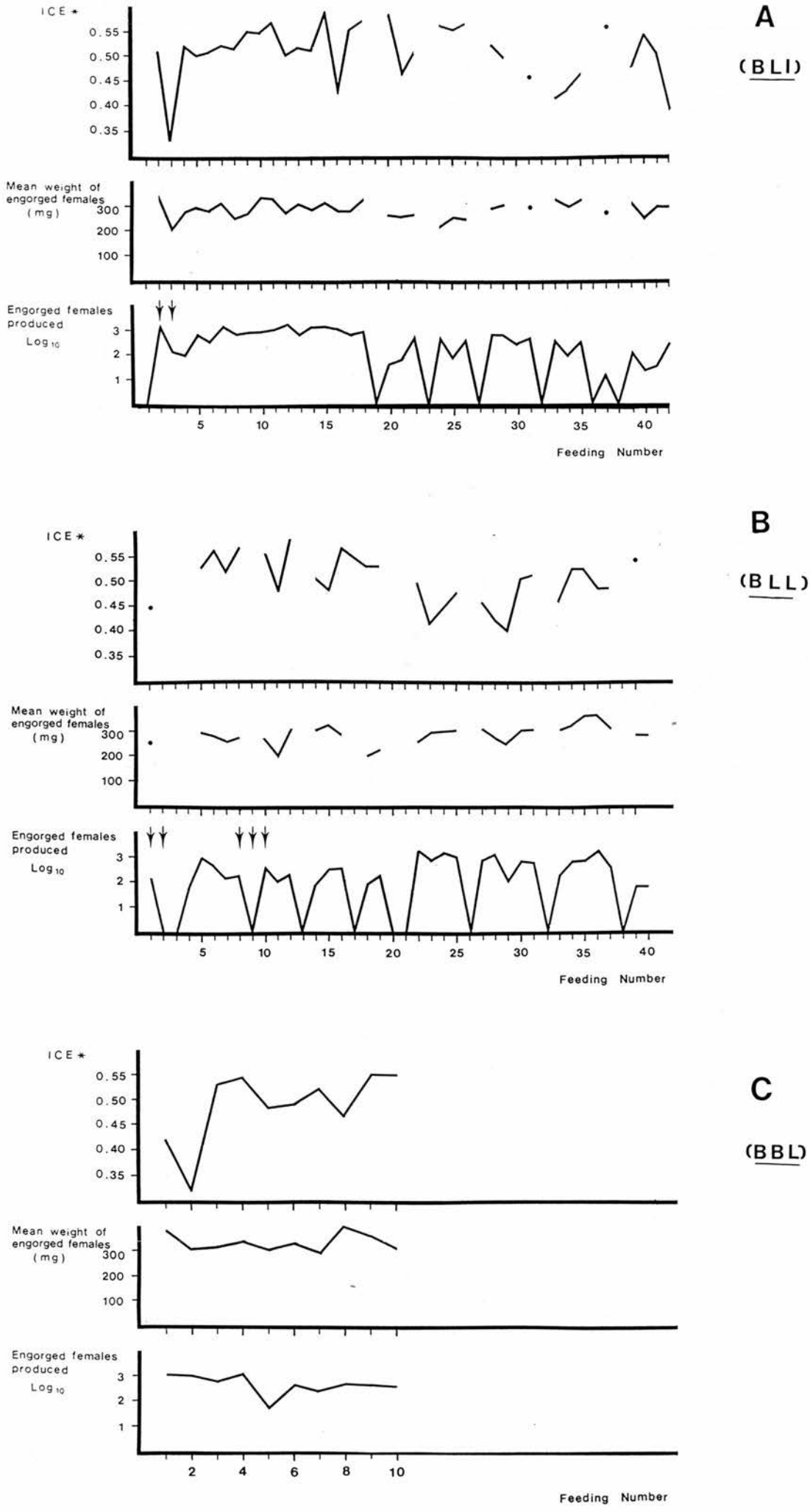
Two calves were used to maintain the colony and were infested alternatively with larvae (usually 10,000) every two weeks. In the productive weeks, naturally detached engorged females were collected twice daily from the floor and walls of the stalls, carried to the

laboratory where they were washed in tap water, and dried on absorbent paper. They were then placed in plastic containers in groups of about 100 females and allowed to oviposit. For this, ticks were placed inside a sealed box, containing a saturated solution of potassium nitrate to a relative humidity of aproximately 80% (Solomon, 1951) and were maintained in an incubator at 28°C.

Two weeks after incubation, the eggs were separated from the ticks (using brush and spatula) and glass tubes containing 0.5, 1.0, 2,0 and 5.0 g of larvae were prepared. It has been stated that one gram of eggs produces approximately 20,000 larvae (Sutherst, Wharton and Utech, 1978). The tubes were sealed with gauze and the eggs allowed to hatch under the same conditions.

Since the beginning of this study the feeding performance of the colony was monitored based on the records for each infestation cycle of the yield of engorged females, the average weight of an engorged female and the Index of Conversion Efficiency (ICE) (Davey et al., 1980). To determine the last two parameters, on each day in which engorged females dropped from the calves (days 19-23 after infestation) 10 ticks were weighed and placed in a separate container. Fourteen days after incubation the eggs produced were weighed to calculate the ICE (ICE = egg mass weight g/weight females g). For each feeding cycle an average of the daily values was calculated (figure 3.1A). This provided quick information on the viability of ticks to be used in the different experiments. After repeated exposure, the donor calves acquired resistance to the tick, impairing the numbers and viability of the ticks produced. In this case the donor calves were changed as quickly as the supply of naive

Figure 3.1. Feeding and ovipositing performance of engorged Boophilus microplus females from three lines of a colony maintained in the laboratory of La Libertad research centre at Villavicencio (A and B) or at LIMV in Bogotá (C). In each line of the colony, the number of engorged females produced in each infestation cycle, their mean weight at engorgement and the Index of Conversion Efficiency (ICE), calculated after 14 days of incubation (ICE = egg mass weight g/weight females g), are indicated. The arrows show the infestation cycles in which the donor calves were treated to control haemoparasitic infection (see text). A, identifies the original line of the colony, that transmitted haemoparasites. B, corresponds to the second line maintained on haemoparasite free calves and treated repeatedly. C identifies the line of the colony fed on a haemoparasite free calf maintained in an isolation unit at LIMV. The discontinuity of the lines in the graph indicate the lack of ticks to measure the parameter on that particular feeding cycle.


A
(BLI)
ICE *
0.55
0.50
0.45
0.40
0.35
Mean weight of engorged females (mg)
300
200
100
Engorged females produced Log10
3
2
1
5
10
15
20
25
30
35
40
Feeding Number
B
(BLL)
ICE *
0.55
0.50
0.45
0.40
0.35
Mean weight of engorged females (mg)
300
200
100
Engorged females produced Log10
3
2
1
5
10
15
20
25
30
35
40
Feeding Number
C
(BBL)
ICE *
0.55
0.50
0.45
0.40
0.35
Mean weight of engorged females (mg)
300
200
100
Engorged females produced Log10
3
2
1
2
4
6
8
10
Feeding Number

animals allowed.

3.1.2.- Procedures to eliminate the infection with haemoparasites from the tick colony.

During the course of this study, various experiments required the use of B. microplus ticks not able to transmit the haemoparasites A. marginale, B. bovis and B. bigemina, which are endemic in the area where the tick colony was maintained. The main purposes of this haemoparasite free tick colony were: the production of tick homogenates without haemoparasitic contaminant proteins, for use on all the purification procedures (4.2.1) and immunological tests (3.6.2); and the production of larvae to infest cattle not protected from haemoparasites, in experiments conducted in the tick-free area of the country (7.2.3).

The following were the procedures to clean the haemoparasitic infection from the tick colony. The calf recipient for the original colony (BLI) was treated with 20 mg/kg Diminazene (4,4'-diamidinodiazoaminobenzene diaceturate: Ganaseg, Squibb, Colombia) on days 12, 15, and 17 and with 20 mg/kg oxytetracycline (Terramicina LA-200, Pfizer, Colombia) on days 15 and 18 post infestation with ticks (Kuttler and Johnson, 1986; Rogers and Dunster, 1984).

Engorged females resulting from this calf were allowed to oviposit and the resulting larvae were used to infest a new pair of calves imported from the tick-free area of the country. These calves were kept in an adjacent enclosure, similar to that described above. This line of the colony was given the code BLL (figure 3.1B). These

animals were continuously monitored for haemoparasitic infection, and treated as above if necessary. Problems were mainly with A. marginale, apparently being transmitted by flies, which could not be entirely precluded from the animal enclosures.

This motivated the creation of a third line of colony at LIMV in Bogotá. There, the tick feeding on calves was performed with less frequency. A calf from the tick free area of the country was used to feed the ticks. This line was coded as BBL (figure 3.1C): It was used as source of ticks in experiments that required haemoparasite free ticks and in the production of the salivary gland homogenates.

3.1.3.- Determination of susceptibility to B. microplus ticks.

Susceptibility to the ticks was measured by counting the number of female ticks 4.5 to 8.0 mm in length ("standard ticks") on the entire animal (Wharton and Utech, 1970). These ticks burdens were the result of either field or artificial infestations with larvae. A gauge was used to facilitate the estimation of sizes of the standard ticks and to train the staff to count ticks (Sutherst and Utech, 1981).

Artificial infestation with ticks was performed using methods similar to those of Utech, Seifert and Wharton (1978). The tubes containing the larvae were covered with gauze and tied to the chest of the animals. The tubes were preferably located under the elbow and left in place for at least two hours, whilst the animals were restrained.

In experiments using large numbers of animals, the ticks for artificial infestation were applied directly on the animals with the

help of a brush whilst the animal was restrained in the cattle crush. In the experiments described in chapters 6, 7 and 8, 10,000 larvae (0.5 grams of eggs) were used for the artificial infestations, but in the final experiment (chapter nine) the number of ticks applied were doubled to increase the reliability of the estimations (Utech, Seifert and Wharton, 1978).

The numbers of ticks surviving to maturity after the artificial infestations were counted from days 19 to 24. However, in the experiment described in chapter six, the tick counts were performed only on days 18, 19 and 20 after infestation due to managerial limitations for the animals. It has been stated that reducing the number of counts does not reduce the accuracy of the test (Sutherst and Utech, 1981).

The levels of susceptibility were calculated as the percentage of larval ticks that succeeded to mature as engorged females assuming a 1:1 sex ratio. (Utech, Seifert and Wharton, 1978).

3.2.- PROTEIN ESTIMATION.

The methods for quantitation of the protein content in solutions of tick-derived proteinaceous materials, changed according to the place of study. Whilst at the CTVM in Edinburgh, a commercial reagent using the bicinchoninic acid method was used (BCA protein assay reagent, Pierce & Warriner Ltd., U.K.). In Colombia, the method of Lowry et al., (1951) was used at first, but proved to be laborious and inaccurate when determining protein at low concentration (under 500 µg protein/ml). Methods for the quantitation of microgram amounts of protein were then adapted to

the equipment available in the laboratory (Spectronic-20, Bausch & Lomb Ltd.).

3.2.1.- The Bradford protein assay method.

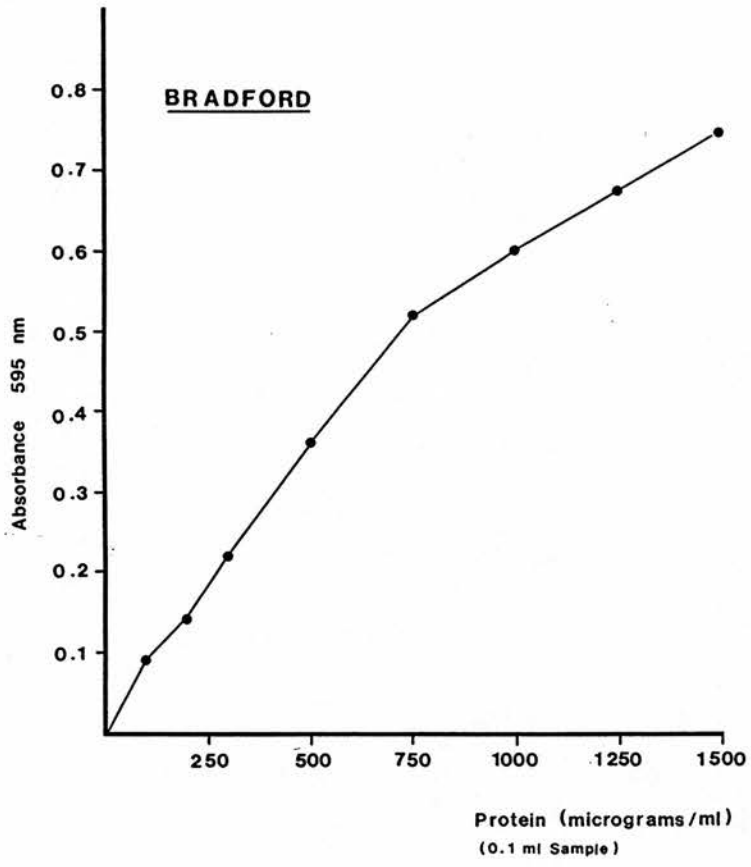
The protein-dye binding assay described by Bradford (1976) was used. The protein reagent was a solution of 0.01% (w/v) coomassie brilliant blue G-250 (Sigma Chemical Co., USA), 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid in distilled water. The sample volume was 0.1 ml and 5 ml of reagent were used. Absorbance was measured immediately at 595 nm. Figure 3.2A illustrates a typical calibration curve using bovine serum albumin. A new curve was calculated for each stock of reagent.

3.2.2.- The Bearden protein assay method.

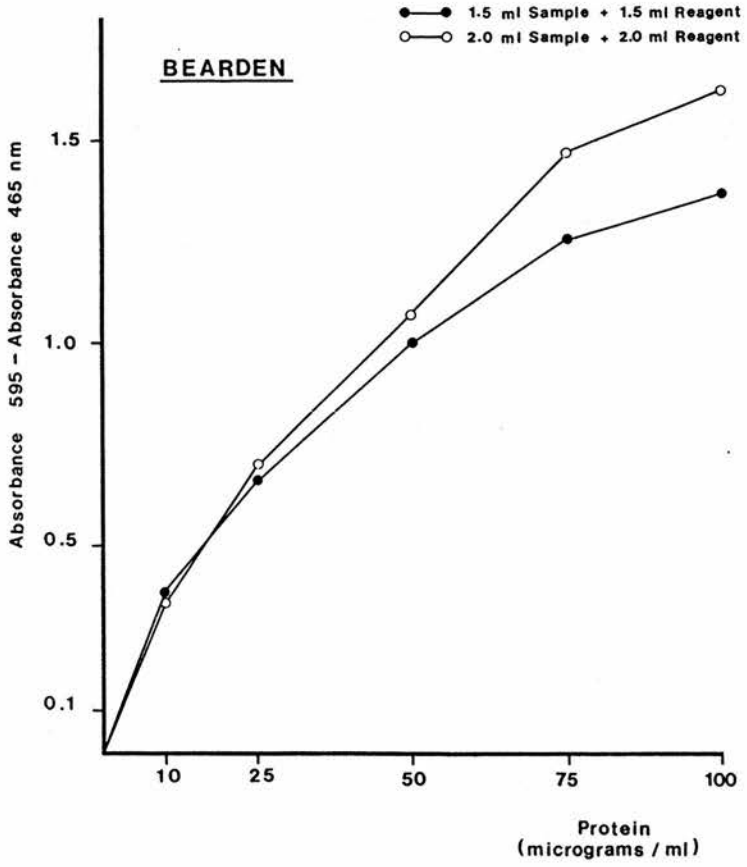
For samples containing protein at low concentration (under 100 µg/ml), the method described by Bearden (1978) was adapted to the laboratory facilities. The protein reagent was a solution of 0.02% (w/v) coomassie brilliant blue G-250 and 17% (w/v) phosphoric acid in distilled water. Equal amounts of sample and reagent were used.

For each sample the absorbance was recorded independently at 465 nm and at 595 nm using as a blank an equal-part mixture of the protein reagent and the buffer in which the proteins were dissolved. The differences between the readings at both wavelengths were calculated by subtracting the reading at 465 nm from the reading at 595 nm. When the absorbance at 465 nm of the sample was found to be below to that of the blank the position of sample and the blank were reversed and the absorbance at 465 nm was added to the reading at

Figure 3.2. Calibration curves to estimate the protein concentration in microgram quantities using two different protein-dye (coomassie brilliant blue G-250) assays. Bovine serum albumin diluted in PBS was used to calculated the curves. Method A (Bradford, 1976) was used to determine protein concentrations ranging between 100 - 1500 micrograms/ml. It used 0.1 ml of sample and 5 ml of reagent and was measured for optical density at 595 nm. Method B (Bearden, 1978) was used to determine protein concentrations ranging between 1 - 100 micrograms/ml. It used either 1.5 or 2.0 ml of both sample and reagent for which both curves are presented. The optical density against the blank was measured at two different wavelenghts and their differences used to plot the graph.


BRADFORD
Absorbance 595 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
250
500
750
1000
1250
1500
Protein (micrograms/ml)
(0.1 ml Sample)

A


1.5 ml Sample + 1.5 ml Reagent
2.0 ml Sample + 2.0 ml Reagent
BEARDEN
Absorbance 595 - Absorbance 465 nm
1.5
1.0
0.5
0.1
10
25
50
75
100
Protein
(micrograms / ml)

B

595 nm. Figure 3.2B illustrates the calibration curves using either 1.5 or 2.0 ml sample volumes.

3.3.- PREPARATION OF SALIVARY GLAND HOMOGENATES FROM FEMALE TICKS.

3.3.1.- Dissection of salivary glands.

The procedure was similar for both, B. microplus and R. appendiculatus ticks. Adult female ticks were allowed to feed (on rabbits for R. appendiculatus or on calves for B. microplus) until the second stage of feeding (Mwangi et al., 1983; Wharton and Utech, 1970). Then they were removed from the hosts and immobilised with their dorsal surface upward in paraffin wax in a Petri dish. The ticks were then covered with a layer of cold PBS (Phosphate Buffered Saline, see below). Using a scalpel blade and forceps, the dorsal integument was removed under a stereoscopic dissecting microscope. The gut diverticula covering the salivary glands were removed and the glands dissected free of the tracheae and other tissues. The salivary glands were then removed after severing the main ducts at their anterior ends, and collected in cold PBS.

3.3.2.- Salivary gland homogenates (GSG).

The salivary glands were homogenized by hand in a glass tissue grinder (Griffiths type) using PBS as diluent (20 pairs of salivary glands/ml PBS). The resulting preparation was filtered twice through a coarse glass fibre filter (millipore type prefilter). The protein concentration was estimated and the resultant solution was stored at

-20°C until required, or freeze dried as described in the respective experiments.

3.3.3.- Phosphate Buffered Saline (PBS).

Phosphate Buffered Saline (PBS), 10 mM pH 7.0, was used as a diluent for proteinaceous materials in the majority of experiments. It was made of 3 mM sodium dihydrogen orthophosphate, 7 mM disodium hydrogen orthophosphate and 127 mM sodium chloride. Solutions were freshly prepared before use.

3.4.- POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

3.4.1.- Separating gel.

Mixtures of proteins were separated by one dimensional electrophoresis in Sodium Dodecyl Sulphate (SDS) on gradient (7-20%) polyacrylamide gels using the discontinuous buffer system of Laemmli (1970). Gels used consisted of a 3 cm stacking gel (total concentration acrylamide + bisacrylamide (T) = 4.5%, 0.1% SDS, 125 mM Tris/HCl pH 6.8) and a 19 cm resolving gel (T = 7-20%, 0.1% SDS, 375 mM Tris/HCl pH 8.8). The electrophoresis buffer was 25 mM Tris, 192 mM glycine and 0.1% SDS pH 8.3).

The samples to be separated were dissolved (1:1 v/v) in a reducing solution (18 mM Tris, 30 mM EDTA, 5% SDS, 2.5% Mercaptoethanol, 25% glycerol and a trace of 0.25% bromophenol blue). They were then heated for five minutes at 100'C, cooled and stored at -20°C until required. Electrophoresis was carried out at 100 volts until the bromophenol blue reached the end of the gel. All

reagents used were analytical grade (Sigma Chemical Co., USA).

3.4.2.- Coomassie blue stain.

In order to visualise the protein bands after electrophoresis the gels were stained with 0.1% (w/v) coomassie blue R-250 (Sigma Chemical Co, USA) in destain solution (25% methanol, 10% glacial acetic acid and 65% distilled water) at room temperature for 2-4 hours. Gels were destained overnight in the destain solution with continuous shaking. Prior to photography, gels were immersed in distilled water for one hour.

3.4.3.- Silver Stain.

For increased sensitivity, gels originally stained with coomassie blue were first destained and then restained using Morrissey's (1981) method for silver staining as follows. The destained gels were incubated in 4% (v/v) glutaraldehyde (EM grade 25%) in 1.9% (w/v) sodium tetraborate solution for 15 minutes in a fume cupboard with occasional shaking. The gels were washed in distilled water (at least 3 x 20 minutes) and then incubated in 0.1% (w/v) silver nitrate (Sigma Chemical Co., USA) for 30 minutes. The gels were rinsed once with distilled water and twice with a developer made of 50 µl of 37% formaldehyde in 100 mls of 3% (w/v) sodium carbonate solution and then left to develop until the desired colour was achieved (5-10 minutes). The reaction was stopped with 10 ml of 2.3 M citric acid, the gels were left in this for one hour and then rinsed in distilled water three times. The gels were photographed soon after developing.

3.4.4.- Calculation of protein Molecular Weights.

Protein molecular weights were calculated by reference to the mobilities of a mixture of low molecular weight calibration proteins (Pharmacia Ltd, U.K.) which included phosphorylase b (94,000 daltons), albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), trypsin inhibitor (20,100 daltons) and alpha-lactalbumin (14,400 daltons).

3.5.- IDENTIFICATION OF TICK ANTIGENS USING WESTERN BLOTTING.

Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose paper at 200 mA, 12 volts for 2 hours in blotting buffer (20 mM Tris/Glycine, pH 9.2 in 20% methanol). When the transfer was complete, the track containing the molecular weight markers was cut off and the proteins visualised by staining in 0.1% amido black (Sigma Chemical Co., USA) in destain solution to check transfer at this point. The rest of the nitrocellulose paper (blot) was placed in blocking buffer (Tris 50 mM, sodium chloride 150 mM, EDTA 1 mM, Nonidet P-40 (Sigma Chemical Co., USA) 0.005%, Gelatin 2.5%, Thimerosal 0.02%, pH 7.4) for four hours to block non-specific protein binding sites on the nitrocellulose paper. For blots using bovine sera, the blocking buffer included 5% skimmed milk (Marvel, Carnation U.K.).

The blot was then incubated overnight in sera of immune animals (either rabbits for experiments using R. appendiculatus antigens, or cattle for experiments using B. microplus antigens; these

corresponded to the reference positive sera used in the ELISA test described below) diluted 1:200 in blocking buffer. A control containing non-immune sera was always included. The blot was then washed in Tris buffered saline (TBS: Tris 20 mM, sodium chloride 500 mM, pH 7.5) with 0.05% Tween-20 over two hours, with a final wash in TBS.

After the washings were completed the blots were incubated with the appropriate horseradish peroxidase-conjugated second antibody (Nordic Immunological Labs., The Netherlands) at a dilution of 1:2000 for the goat anti-rabbit immunoglobulin G (GAR-IgG/PO) or at 1:1000 for the goat anti-bovine immunoglobulin G (GAB-IgG/PO) in blocking buffer. After this, the blot was washed in seven changes of TBS with 0.05% Tween-20 for 5 minutes each change and finally washed twice in TBS to remove Tween-20.

The position of antigen-antibody complexes were detected by incubating the blot in a substrate freshly made by adding 20 ml of 4ClN (4-chloro-1-naphthol (Sigma) dissolved at 3 mg/ml in ice cold anhydrous methanol) to 100 ml TBS and 60 microlitres of 30% hydrogen peroxide, until colour developed. The blot was finally rinsed in purified water.

3.6.- ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

The ELISA was used to study the production of anti-tick antibodies in animals exposed to ticks in either of the two systems studied; R. appendiculatus-rabbits or B. microplus-cattle. An indirect test for quantitation of antibody (Voller and de Savigny, 1981) was standardized for each tick-host system. In each of them, a

salivary gland homogenate (GSG) from the respective tick species was used as antigen.

3.6.1.- General protocol for the test.

The protocol was similar to that described by Njau and Nyindo (1987). Microtiter plates (Immulon 2, Dynatech Labs., USA) were coated overnight at 4°C with the appropriate amount of antigen (3.6.2) diluted in carbonate-bicarbonate buffer pH 9.6 (15 mM sodium carbonate, 35 mM sodium bicarbonate).

The antigen coated plates were later rinsed 3 times for 3 minutes each, with washing solution (137 mM sodium chloride, 1 mM potassium dihydrogen orthophosphate, 8 mM disodium hydrogen orthophosphate, 3 mM potassium chloride and 0.05% Tween-20 pH 7.2) and then the non-specific receptor sites were blocked by filling all the wells with washing solution containing 0.3% (w/v) pig gelatin (blocking buffer solution), and the plates incubated 2 hours at 25°C.

Samples of 180 µl of test sera appropriately diluted in blocking solution were added to each well and incubated at 37°C for 30 minutes (control wells filled with blocking solution were included in all the plates). The plates were then rinsed 3 times (as above) with washing solution to remove unbound antibodies.

After this, 200 µl of the corresponding horseradish peroxidase-conjugated antisera (conjugate) were placed in all wells and incubated at 37°C for 30 minutes. For the experiments using rabbit sera, the conjugate was GAR-IgG/PO diluted at 1:4000 in blocking solution (Fletcher J., personal communication, 1988). For

experiments using bovine sera, the conjugate was GAB-IgG/PO diluted at 1:1000 in blocking solution (Walker, Fletcher and Todd, 1989).

After incubation, the plates were washed as above and then all the wells were filled with 200 µl of a freshly made solution of chromogen and substrate. This solution was made by adding the chromogen (10 mg Tetramethyl benzidine dissolved in 1 ml of dimethyl sulphoxide) to 99 ml of 100 mM acetate-citrate buffer pH 6.0, and to this the substrate (15 µl of hydrogen peroxide 30%) was added. The plates were kept in darkness at 25°C for 30 minutes. After this the reaction was stopped with 50 µl of 2 M sulphuric acid added to each well and the optical density was determined at 450 nm with an ELISA plate reader (Titertek Multiskan, Flow labs., U.K.).

Absorbance values obtained on different days were corrected with reference to a standard positive sera, this was included in all the plates, using the following correction formulae (Voller and Bidwell, 1986):

$$\text{Corrected Absorbance} = \frac{\text{Abs. of sample} \times 0.8}{\text{Abs. standard positive}}$$

3.6.2.- Concentration of antigen used in the ELISA test.

Experiments with rabbit sera used GSG from *R. appendiculatus* females as antigen. The working concentration of antigen was that reported by Walker, Fletcher and Todd (1989), which corresponded to 20 ng of protein/well. Experiments with bovine sera used GSG from *B. microplus* females as antigen. The optimal concentration of this antigen was determined after testing the responses of known positive and negative sera (see below) diluted in two-fold serial dilutions

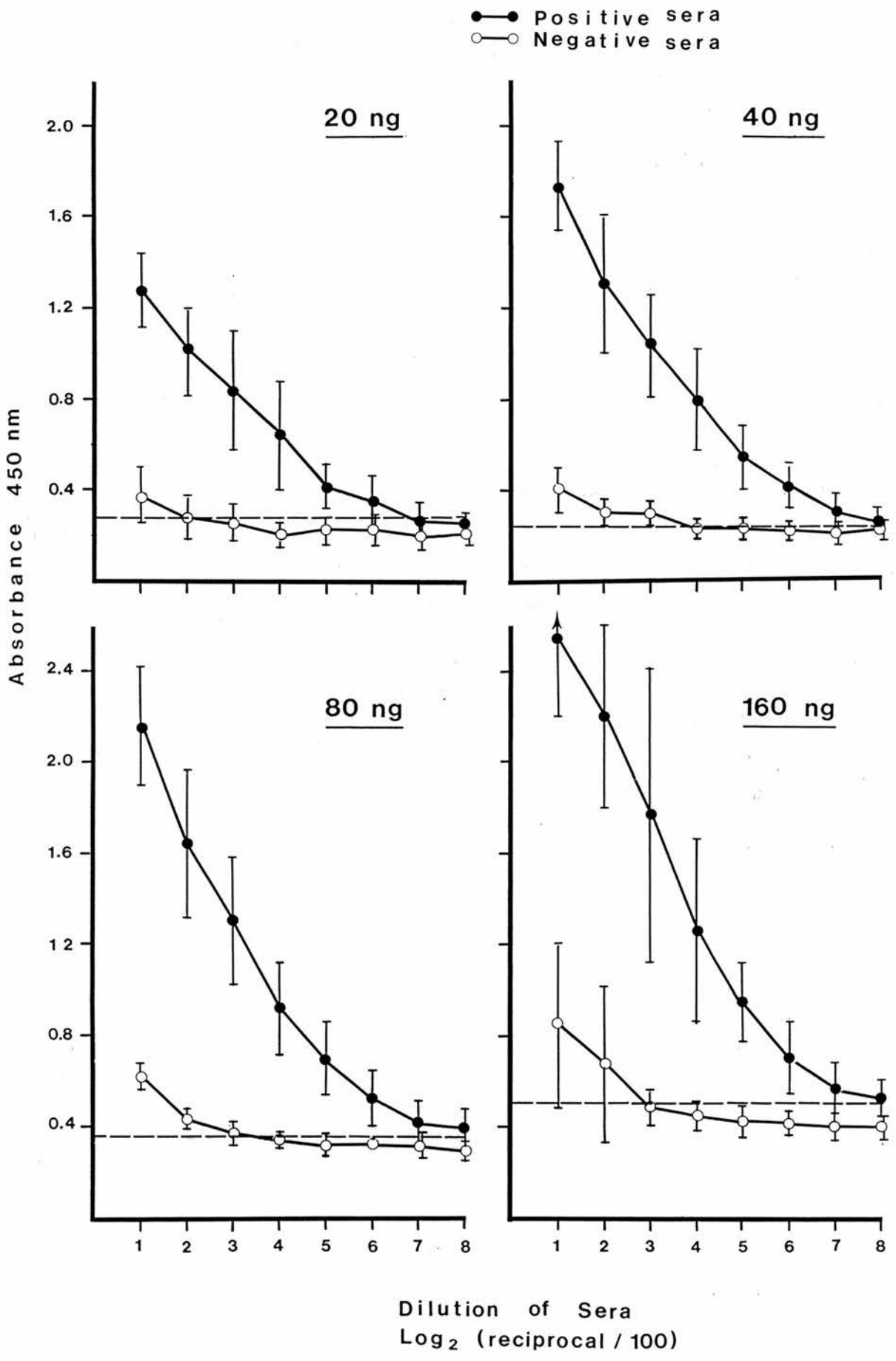
starting at 1:200, on plates containing antigen in concentrations of 20, 40, 80 and 160 ng of protein/well (figure 3.3). Based on those responses using the criteria suggested by Voller and Bidwell (1986) and on considerations of economy of antigen, the 40 ng/well concentration of antigen was chosen.

3.6.3.- Positive and negative reference sera and threshold cut-off for positive values.

Sera from rabbits exposed at least once to all instars of the tick R. appendiculatus were used as reference positive in experiments using GSG from R. appendiculatus. Sera from rabbits naive to the tick were used as reference negative. With this tick and host, the threshold value was calculated by reference to the titration curve of a group of 10 negative sera. It was set at an absorbance value of 0.3, the doubled value of the mean observed in the group of negative sera at a dilution of 1:800, point at which the titration for the negatives showed to approach the flat tail of the sigmoid curve (Kurstak, 1985). The mean for the negative sera at the dilution of 1:200 was 0.246.

In experiments with B. microplus and cattle, the positive standard sera were selected from those producing the highest readings on preliminary tests using 70 ng antigen/well, on which, sera from cattle repeatedly exposed to B. microplus ticks and displaying resistance were tested. Forty sera from healthy tick-naive calves were used as negative controls. These sera were collected from animals both at the Tibaitatá research centre in Bogotá or at CTVM in Edinburgh.

Figure 3.3. Titration of known positive and negative sera to determine the optimal concentration of antigen in an ELISA test using a salivary gland homogenate from Boophilus microplus females. The concentrations of antigen assayed were 20, 40, 80 and 160 ng protein per well, all included in the same plate. Five repeats were studied. Sera were initially diluted to 1:200 (1) and then on two-fold steps up to 1:25,600 (2-8). The bound antibodies were detected using GAB-IgG/PO and tetramethyl benzidine as a chromogen. Optical density was measured at 450 nm. Vertical bars indicate standard errors.


Positive sera
Negative sera
20 ng
40 ng
80 ng
160 ng
Absorbance 450 nm
2.0
1.6
1.2
0.8
0.4
2.4
2.0
1.6
1 2
0.8
0.4
1 2 3 4 5 6 7 8
Dilution of Sera
Log2 (reciprocal / 100)

The threshold value was calculated again by reference to a titration curve of the group of negative sera. A calf from this group producing high readings, (outside the range of the mean + 3 standard deviations) was eliminated for the calculations. The threshold value was set at an absorbance of 0.24 (mean + 2 standard deviations at 1:800). The mean absorbance value for the bovine negative sera at the lowest dilution (1:200) was 0.19.

3.6.4.- Calculation of endpoint titres using a reference curve.

The amount of specific antibodies contained in the sera were presented as endpoint titres on a continuous scale. For this the titre was expressed as the reciprocal of the dilution used, which was indicated as the base 2 logarithm of the dilution used divided by 100. For example, the titre for dilution 1:200 was 1, for dilution 1:400 was 2, and so on.

In experiments with rabbits, since small numbers of samples were studied, the endpoint titres were calculated by a graphic plot of the optical density of each sera serially diluted using two-fold steps, starting at 1:200. The point at which the curve for each individual, intersected the cut-off point (0.3) was presented as the endpoint titre on a continuous scale (Voller and de Savigny, 1981).

In experiments with cattle, the endpoint titres were calculated with reference to a standard curve (Kurstak, 1985; Voller and de Savigny, 1981) using the absorbance values obtained for each sera at a fixed dilution of 1:200. To produce the standard curve, sera from 68 cattle (including strongly and weakly positive and negative animals) were titrated and the dose-response curves of the optical

density were individually plotted for each animal, to obtain the endpoint titre value. These values were then re-plotted as a function of the ELISA value (absorbance) obtained at the 1:200 dilution (figure 3.4).

3.7.- PROCESSING OF SKIN BIOPSIES AND CELL COUNTS.

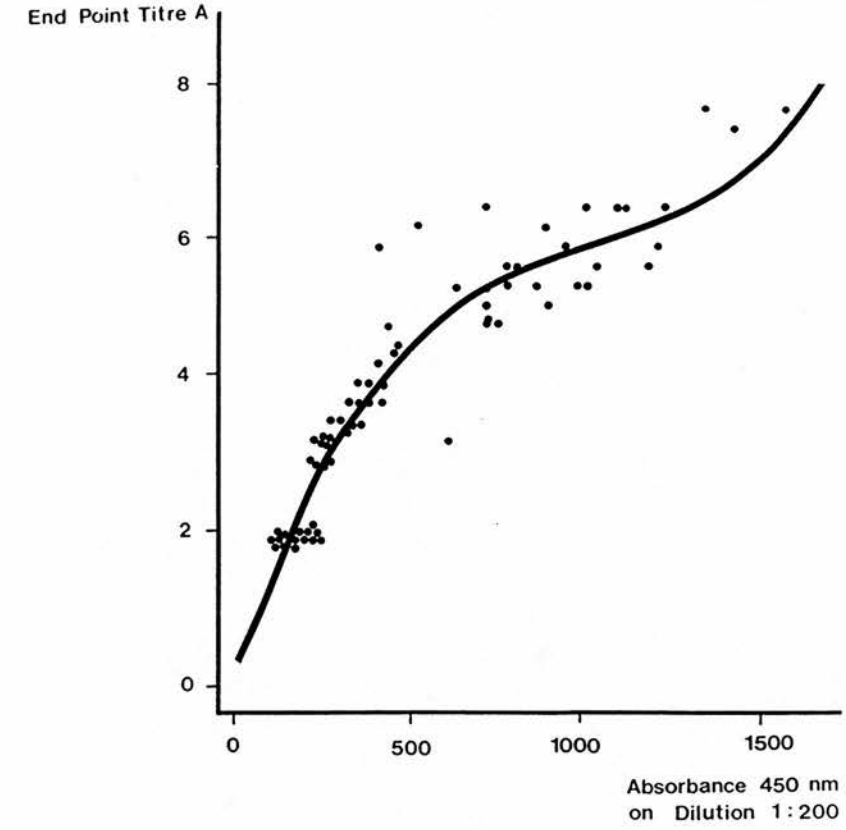
Biopsies of skin test reactions, at various times after inoculation in the skin of rabbits were conducted using methods similar to those of Walker and Fletcher, (1987). The area around the lesion was infused with local anaesthetic (2% Xylocaine) and biopsies were taken using a 3.5 mm trephine which rotated rapidly with a hand-held electric motor. Biopsies were immediately placed in Karnovsky's fixative (2.5% (w/v) glutaraldehyde, 2.0% (w/v) formaldehyde in 100 mM phosphate buffer pH 7.2) at 4°C for 4 hours. The samples were then washed in 100 mM phosphate buffer and embedded in hydroxyethyl methacrylate using a commercial kit (Historesin, LKB, Sweden). The embedded biopses were sectioned at 1 and 2 µm using glass knives on a Cambridge-Huxley Ultramicrotome (Cambridge Scientific Instruments, U.K.) and stained with Giemsa's stain diluted 1:10 in 100 mM acetate buffer, pH 4.5.

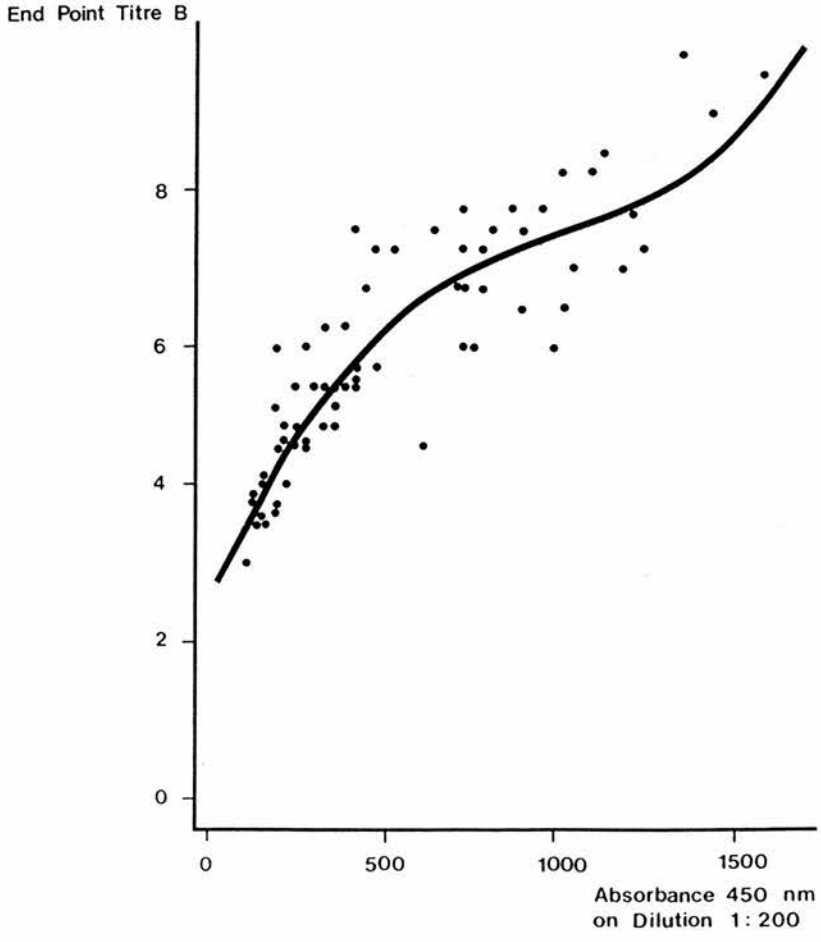
By light microscopy, the cells encountered in 10 oil fields (x 1000, 0.24 mm^2 total area) were counted and differentiated by the criteria suggested by Walker and Fletcher (1986).

3.8.- TECHNIQUES FOR DIALYSIS AND CONCENTRATION OF PROTEINS.

Protein solutions were dialysed using cellulose dialysis membranes as tubing (Spectra/Por 2, MWCO. 12,000-14,000; Spectrum

Figure 3.4. Standard curve to calculate the endpoint titre of sera based on the optical density at the working dilution (1:200). Calculations using two different cut-off values to discriminate between positives and negatives are presented to illustrate the changes in titre related to the use of different criteria. Endpoint titre A was calculated using as a cut-off point, a value of absorbance of 0.24. Endpoint titre B was calculated using a cut-off value of 0.1. To produce the curves, the endpoint titre for each sera was calculated using the dose-response curve of the optical density after titration. Then these values were re-plotted as a function of the optical density observed for each sera at the 1:200 dilution.


End Point Titre A
8
6
4
2
0
0
500
1000
1500
Absorbance 450 nm
on Dilution 1:200


End Point Titre B
8
6
4
2
0
0
500
1000
1500
Absorbance 450 nm
on Dilution 1:200

Medical Ind., USA), which after being sealed were immersed in at least 10 volumes of the appropriate buffer under continuous stirring at 4°C.

Protein concentration was conducted either by placing the dialysis bags containing the protein solution on dry polyethyleneglycol compound (Sigma Chemical Co., USA) until the desired concentration or by ultrafiltration using a standard stirred cell and a YM-5 Amicon membrane (Amicon Co., USA) at a pressure of 10 psi.

3.9.- GENERAL STATISTICAL METHODS.

Data collected from field and laboratory experiments were stored and manipulated for statistical analysis with the help of a computer using a commercial software (PANACEA, PAN Livestock Services Ltd., U.K.). Some remarks are required on the assumptions and the steps followed for the analysis.

The normality of the distribution was primarily tested for each parameter using the histogram command of the software, which allowed to test the goodness-of-fit to a normal distribution (Bailey, 1981). However, in some experiments it was considered more appropriate to test the data for normality using a graphic plot of the observed values, using rankits (= normal order statistics) (Sokal and Rohlf, 1981).

Analysis of variance (ANOVA) was conducted exploiting the features of the software. A Bartlett's test for the homogeneity of variances is conducted as the first part of the analysis (Bailey, 1981). The significance of the difference between the means of the

various groups is tested by the software using Student's 't' test between adjacent means arranged in ascending order. This test was used as a rough guide, but a Duncan's multiple range test (Little and Hills, 1976) was calculated by hand, based on the output of the analysis. Two-way ANOVA was conducted similarly and when required data were rearranged and tested as one-way ANOVA.

Multiple linear least-squares regression analysis and the preparation of the multiple matrix of correlations between set of variables were conducted using a multiple regression command of the software. In the case of multiple regression it is not necessary that any of the variables used in the analysis follow a normal or any other distribution. In the software the estimated regression coefficients and the regression itself are tested for significance and the multiple coefficient of determination is displayed (Bailey, 1981).

Non-parametric statistics were calculated by hand, but using the indexing and sorting facilities of the software.

CHAPTER FOUR:

PARTIAL PURIFICATION AND CHARACTERIZATION OF TICK-DERIVED PROTEINS USING CHROMATOGRAPHIC METHODS

SUMMARY

Methods of fractionation by precipitation on salt saturated solutions and of anion-exchange chromatography were used to separate proteins contained in crude tick-derived extracts either from larvae or from salivary glands of females. These methods were refined in Edinburgh (CTVM laboratory) working with Rhipicephalus appendiculatus ticks and then were implemented in Bogotá, working with Boophilus microplus ticks. The degree of separation of proteins obtained with the use of these methods was studied by the comparison of the electrophoretic pattern on polyacrylamide gels and by the identification of immunoreactive molecules using the Western blotting technique. The similarity of results obtained with different ticks species and at different laboratories is discussed.

4.1.- INTRODUCTION.

The fact that cattle exposed to Boophilus microplus ticks display dermal hypersensitivity reactions to the inoculation of tick derived proteins has been recognized for many years. The mechanisms of these reactions and their relationship with the resistance to the tick by the cattle have been widely investigated. During those studies, materials used for the inoculations have included: crude extracts of tick eggs or larvae (Riek, 1962) and tick saliva

obtained by pilocarpine stimulation (Tatchell, 1969), as well as highly purified antigens separated from larval extracts (Willadsen and Williams, 1976; Willadsen et al.,1978; Willadsen and Riding, 1979). Two of those purified antigens when tested on cattle with differing degrees of resistance to the tick showed correlation between the sensitivity to the antigen and the level of immunity of the animals to the tick (Willadsen et al., 1978).

Although Willadsen (1980b) states that the use of crude extracts of allergenic materials can cause misleading results because the specific immunological reactions are mixed with non-specific skin inflammation there are many problems related with the use of purified materials when attempting their use for widespread practical purposes (Walker and Fletcher, 1989). Also the problems with the application of modern technologies in third world countries have been discussed in the introductory chapter.

During the present study it was attempted to produce partially purified antigens from tick-derived materials, (in conditions and quantities suitable for field use). These were used as antigens in skin tests to study the relationship between the resistance of the animals to the tick and the skin response to the antigens.

A simplification of the purification procedures described by Willadsen and Williams (1976) was used. Preliminary chromatographic procedures were peformed at the CTVM in 1983 using larval extracts and salivary gland homogenates of Rhipicephalus appendiculatus ticks, readily available at the Centre, These methods were then used on B. microplus larval extracts. On return to Colombia the chromatographic work was performed using the facilities available at

the immunology section of the LIMV at Bogota, using B. microplus ticks.

4.2.- MATERIALS AND METHODS.

4.2.1.- Ticks.

Larvae and females R. appendiculatus ticks were obtained from the laboratory colony maintained at the CTVM. All these ticks were derived from ticks fed on rabbits. B. microplus larval ticks used during the preliminary experimentation at the CTVM were kindly supplied by Mr. M. Matthewson (Coopers Animal Health Ltd, England). The chromatographic separations performed at LIMV used B. microplus larvae from the haemoparasite free colony described in chapter three. For the preparation of the extracts, ticks not older than 20 days after hatching were used.

4.2.2.- Preparation of crude extracts

4.2.2.1.- Preliminary developments at the CTVM

Initial trials were performed using R. appendiculatus larvae and similar methods were later applied to B. microplus larvae. The methods for the preparation of the larval extracts were a simplification of those used by Willadsen and Williams (1976), and are summarized in figure 4.1. Each preparation used different amounts of larvae and all procedures were conducted at 4°C.

Two different protocols were used to clarify the supernatant. Protocol A, was used initially in the preparation of extracts RAPP-1 and RAPP-2 (table 4.1), but it was later simplified to protocol B,

Figure 4.1. Methods for the preparation of crude larval extracts from either Rhipicephalus appendiculatus or Boophilus microplus ticks and methods for their subsequent fractionation by precipitation at different concentrations of ammonium sulphate. a) Preparation of the crude larval extracts. Different ammounts of larvae were used to prepare each extract. All procedures were conducted at 4°C.

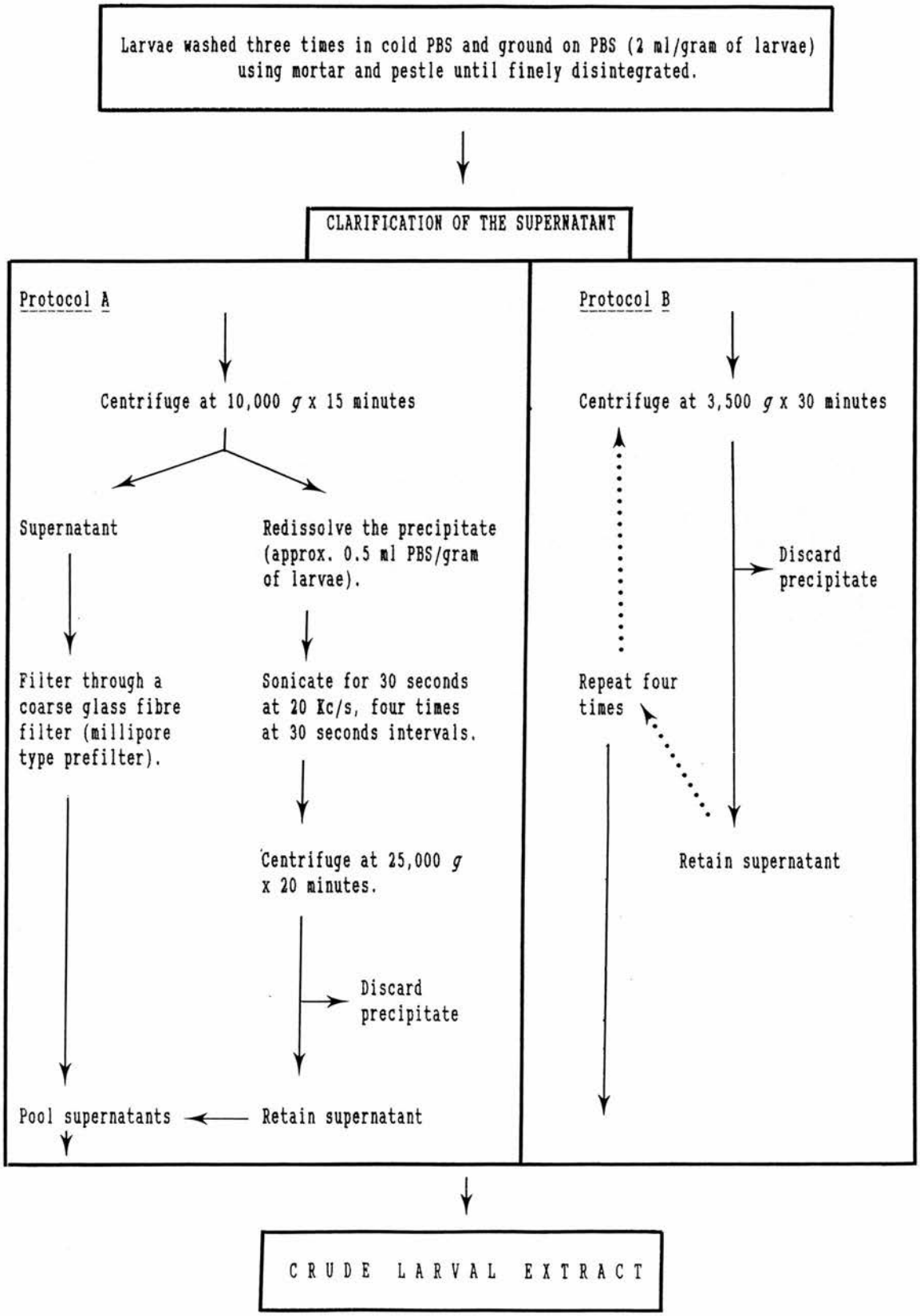

Larvae washed three times in cold PBS and ground on PBS (2 ml/gram of larvae) using mortar and pestle until finely disintegrated.
CLARIFICATION OF THE SUPERNATANT
Protocol A
Centrifuge at 10,000 g x 15 minutes
Supernatant
Redissolve the precipitate (approx. 0.5 ml PBS/gram of larvae).
Filter through a coarse glass fibre filter (millipore type prefilter).
Sonicate for 30 seconds at 20 Kc/s, four times at 30 seconds intervals.
Centrifuge at 25,000 g x 20 minutes.
Discard precipitate
Pool supernatants
Retain supernatant
Protocol B
Centrifuge at 3,500 g x 30 minutes
Discard precipitate
Repeat four times
Retain supernatant
CRUDE LARVAL EXTRACT

Figure 4.1. (continuation).
b) Fractionation by precipitation at different concentrations of ammonium sulphate. The salt was slowly added to the protein solution under continuous stirring until the desired concentration. During the purifications performed at LIMV in Colombia a 65% saturation of ammonium sulphate was used for the second precipitate (65% ASP).

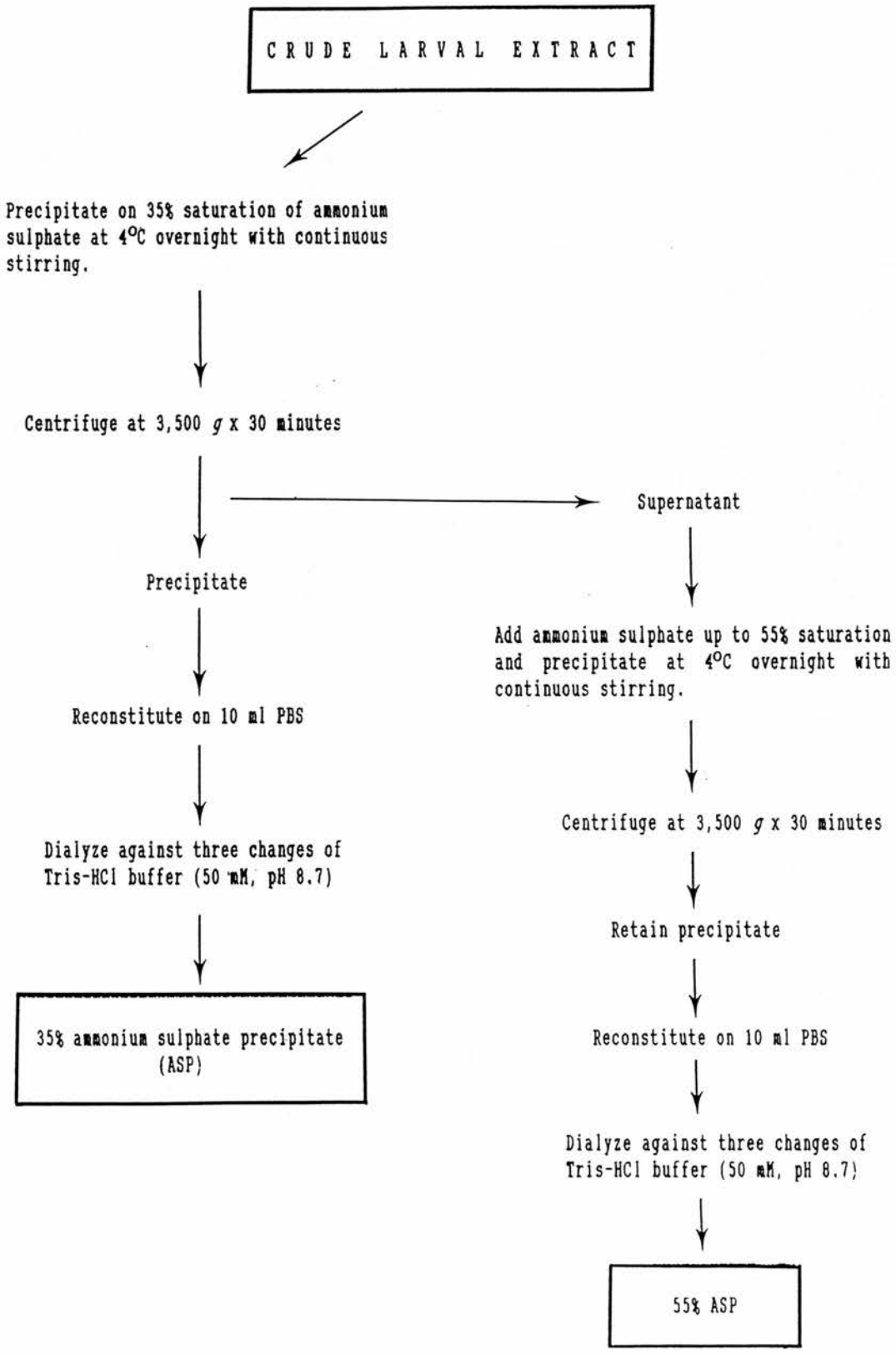

CRUDE LARVAL EXTRACT
Precipitate on 35% saturation of ammonium sulphate at 4°C overnight with continuous stirring.
Centrifuge at 3,500 g x 30 minutes
Precipitate
Reconstitute on 10 ml PBS
Dialyze against three changes of Tris-HCl buffer (50 mM, pH 8.7)
35% ammonium sulphate precipitate (ASP)
Supernatant
Add ammonium sulphate up to 55% saturation and precipitate at 4°C overnight with continuous stirring.
Centrifuge at 3,500 g x 30 minutes
Retain precipitate
Reconstitute on 10 ml PBS
Dialyze against three changes of Tris-HCl buffer (50 mM, pH 8.7)
55% ASP

Table 4.1.- Preparation of extracts from Rhipicephalus appendiculatus and Boophilus microplus ticks at CTVM

		TOTAL PROTEIN CONTENT (mg)		
Extract code 1	Ammount 2	Crude extract mg	35% ASP mg *	55% ASP mg *
RAPP-1	1.5	150	20 R1-C1	47 R1-C2
RAPP-2	6.0	350	87 R2-C1	75 R2-C2
RAPP-3	2.0	N.D.	N.D.	32 R3-C2
BOOM-1	3.0	330	26 B1-C1	52 B1-C2
BOOM-2	5.0	290	51 B2-C1	44 B2-C2
GSG-1	100	130	6 G1-C1	54 G1-C2
GSG-2	150	2.28	16 G2-C1	11 G2-C2

* : Percentage of saturation of ammonium sulphate used to precipitate the protein. The codes identify the column to which each extract were applied for chromatography.

1 : RAPP - extracts from R. appendiculatus larvae.
BOOM - extracts from B. microplus larvae.
GSG - salivary gland homogenates from R. appendiculatus females.

2 : Grams of larvae (RAPP, BOOM) or pairs of salivary glands (GSG).

N.D. Not done.

to facilitate its use on return to Colombia (applied to prepare extracts RAPP-3, BOOM-1 and BOOM-2). Crude extracts were fractionated for protein by differential precipitation with ammonium sulphate at concentrations of saturation of 35% and 55% (as described in figure 4.1). Two protein fractions were so obtained from each extract, and were named as 35% ammonium sulphate precipitate (35% ASP) and as 55% ASP. They were either used immediately for chromatographic separation or frozen at -20°C until required.

Similar methods were used to fractionate salivary gland homogenates (GSG) from R. appendiculatus semi-engorged females. GSG were prepared as described in chapter three (3.3) using 100 to 150 pairs of salivary glands, and sonicated (for 30 seconds at 20 Kc/s, four times at 30 seconds intervals). The homogenates were then centrifuged twice as in protocol B and fractionated for proteins with ammonium sulphate using methods as those described for larval extracts.

Table 4.1 summarizes the observations on these preliminary developments using either R. appendiculatus (RAPP), B. microplus (BOOM) larval extracts, or GSG. Each of the fractions obtained with the methods described above was identified with a code indicating: the source of materials (R for RAPP larvae or B for BOOM larvae, and G for GSG), the numeric order of the crude extract and the concentration of ammonium sulphate in which it was fractioned (-C1 for 35% and -C2 for 55%). These codes were used later to identify the columns for the chromatographic separation of the respective materials.

4.2.2.2.- Preparation of B. microplus larval extracts at LIMV.

A total of 10 larval extracts based on large ammounts of larvae were prepared during the period of study at Colombia. Methods used for their preparation were similar to those described above (only protocol B was used) with the adition that the extracts were filtered through gauze before the initial centrifugation. The fractionation of proteins on ammonium sulphate was performed at concentrations of saturation of 35% and 65%, to correspond closely to described methods (Willadsen and Williams, 1976). A slight precipitation of protein was found during dialysis and was removed by centrifugation, before determining the protein content (see below). Crude larval extracts were prepared and kept frozen until required for ammonium sulphate precipitations. These precipitations were usually performed on two or three different extracts at a time (before the commencement of a chromatography) and fractions not used immediately were frozen again.

Precipitation of protein was found on some frozen extracts due to problems faced with the freezing equipment of the laboratory. (Due to the scarcity of space, materials were kept in various freezers in different sections of the laboratory). These extracts were centrifuged and protein concentration assayed before use. Extracts that showed extensive precipitation were discarded. A summary of these activities is shown in table 4.2.

4.2.3.- Separation of tick homogenates using anion-exchange chromatographic techniques.

Table 4.2.- Preparation of extracts from B. microplus larvae and fractionation on different concentrations of saturation of ammonium sulphate. Work performed at LIMV, 1986 - 1988.

			TOTAL PROTEIN CONTENT (mg)		
Extract	Date of preparation	Ammount of larvae (grams)	Total extract	35% ASP @	65% ASP @
L1	14-11-86	50	N.D.*	N.D.	N.D.
L2	16-01-87	74	626	111	163
L3	2-03-87	11	318	139	88*
L4	14-04-87	83	1044	217	550
L5	21-04-87	40	159	62*	35*
L6	5-06-87	30	476	107	247
L7	16-07-87	66	753	184	254
L8	25-09-87	120	N.D.*	N.D.	N.D.
L9	25-01-88	58	N.D.	144	220
L10	11-08-88	89	3200	158	2258

* = Indicates material damaged by deficiencies on the freezing equipment.

@ = Concentration of saturation of ammonium sulphate used to precipitate the protein on the corresponding fraction.

N.D. = Not done

4.2.3.1.- Preliminary developments at the CTVM

Methods similar to those described by Willadsen and Williams (1976) were followed. During the period of study at Edinburgh, anion-exchange chromatography of the extracts was performed using a 1.6 x 40 cm column of diethylaminoethyl (DEAE) cellulose (DE-52, Whatman Ltd.) equilibrated with Tris-HCl buffer (50 mM, pH 8.7). The initial chromatographic separations used a stepwise change in the molarity of sodium chloride in the eluting buffer (0, 50, 75, 100, 150 and 200 mM), but later the procedure was changed to a 500 ml linear gradient from 0 to 300 mM sodium chloride.

Protein peaks eluted from the columns were observed as the increases in the absorbance at 280 nm. Fractions of corresponding peaks were pooled and concentrated by dialysis against solid polyethylene glycol (Sigma Chemical Co., Ltd) down to a final volume of 2-5 mls. Each fraction was desalted by dialysis against distilled water, the protein content estimated and then freeze dried.

Whilst at Edinburgh, chromatographic separations were attempted on larval extracts from R. appendiculatus and B. microplus and on salivary gland homogenates from R. appendiculatus females.

4.2.3.2.- Chromatographic separations at LIMV.

At LIMV chromatographic separations were performed on a 2.8 x 45 cm column of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals Inc., USA) equilibrated on Tris-HCl buffer (50 mM, pH 8.7), and for the elution, a 600 ml linear gradient of sodium chloride from 0 to 500 mM on the same Tris-HCl buffer was used.

Eluted peaks were concentrated and desalted either as described above or using ultrafiltration membranes (Amicon YM-5, Amicon Co., USA). The protein concentration was then determined and the materials were aliquoted and frozen. For the last set of field experiments (chapter nine) and for the transport of materials to Edinburgh in the final period of study, aliquoted materials were freeze dried (with the generous collaboration of VECOL laboratories, Bogotá).

4.2.4.- Protein Estimation.

During the period of study at Edinburgh, the protein concentration of the crude extracts were determined using the method of differential optical absorbance at 280 and 260 nm (Layne, 1957) and on concentrated peaks using the BCA Protein Assay reagent (Pierce & Warriner Ltd., U.K.).

In Colombia, a dye-binding assay (Bradford, 1976) was used to estimate the protein concentration both on the crude extracts and on the concentrated peaks. A more sensitive modification of the dye-binding assay (Bearden, 1978) was implemented for materials of low concentrations (under 100 micrograms protein/ml). The standardization of these techniques is described in chapter three (3.2).

4.2.5.- Partial characterization of the separated proteins using gel electrophoresis and the recognition of antigens by immune sera.

The composition of the protein peaks eluted from the columns

was studied using SDS-PAGE (3.4). Protein bands observed were compared among the different isolates using the index of similarity of Lawson et al. (1980), which is the inverse of the percentage of protein bands that are not common between the gels being compared. Immunogenicity of the protein contained on the fractions was studied by an immunoblotting technique (3.5).

4.3.- RESULTS

4.3.1.- Fractionation of proteins from R. appendiculatus larval extracts and salivary gland homogenates.

Results of the fractionation of larval homogenates are summarized in figure 4.2. It was found consistently that four protein peaks were eluted from the column in response to the increasing molarity of sodium chloride used in the eluant. They were given the following nomenclature: Peak 1 (PK1) = proteins eluted at 50 mM sodium chloride, Peak 2 (PK2) = proteins eluted at 75 to 100 mM sodium chloride, Peak 3 (PK3) = proteins eluted at 150 mM sodium chloride and Peak 4 (PK4) = proteins eluted at 200 mM sodium chloride.

Materials obtained from column R1-C2 were used in the preliminary experiment on skin test methods in rabbits (5.2.4.1). The proteinaceous composition of each of the fractions was studied using SDS-PAGE. Figure 4.3 shows the proteins contained on each peak, these results were consistent for all the chromatographies performed. The components of PK1 and PK2 had very low concentration of protein, and thus were only visualized using the silver stain

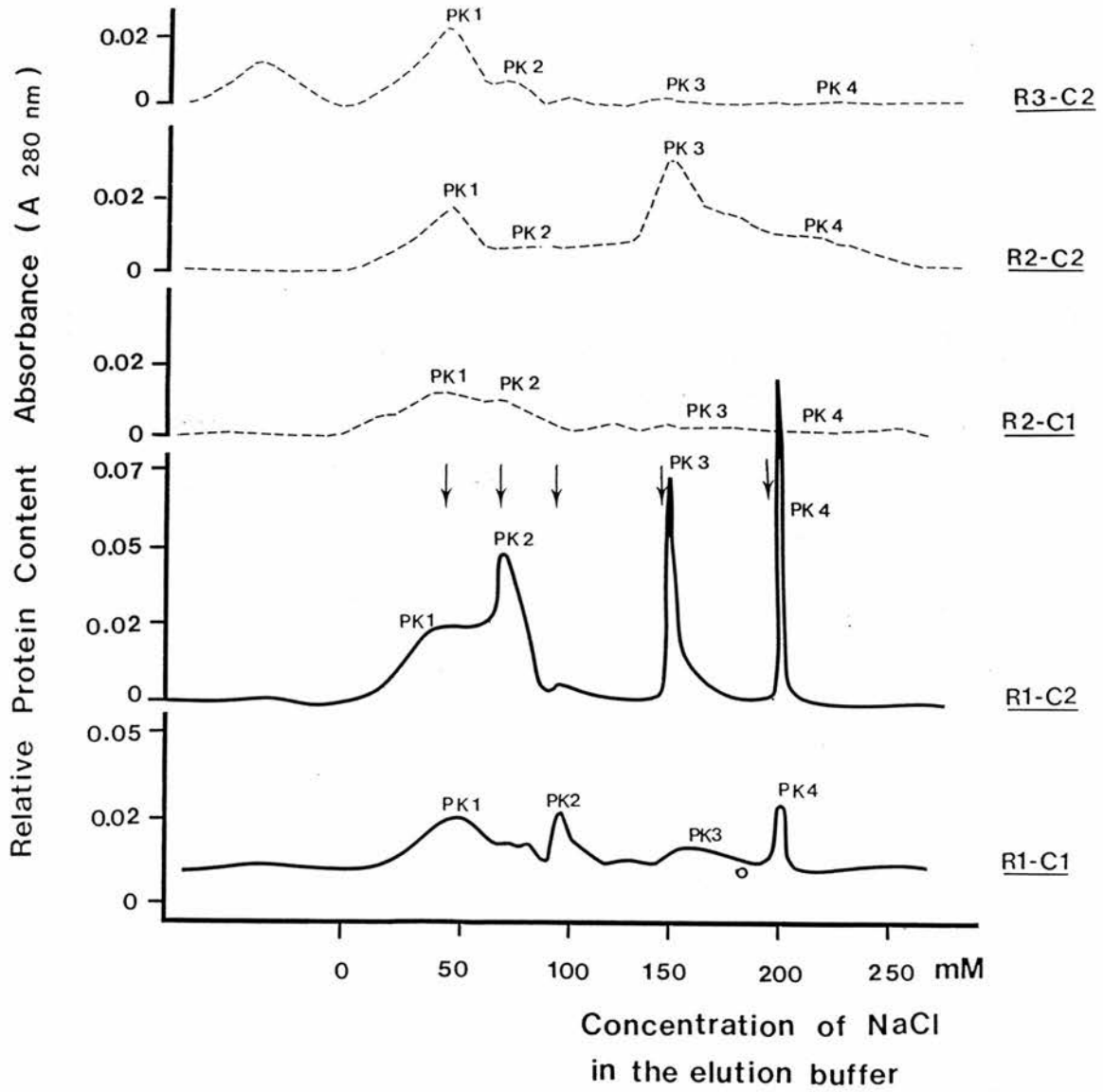
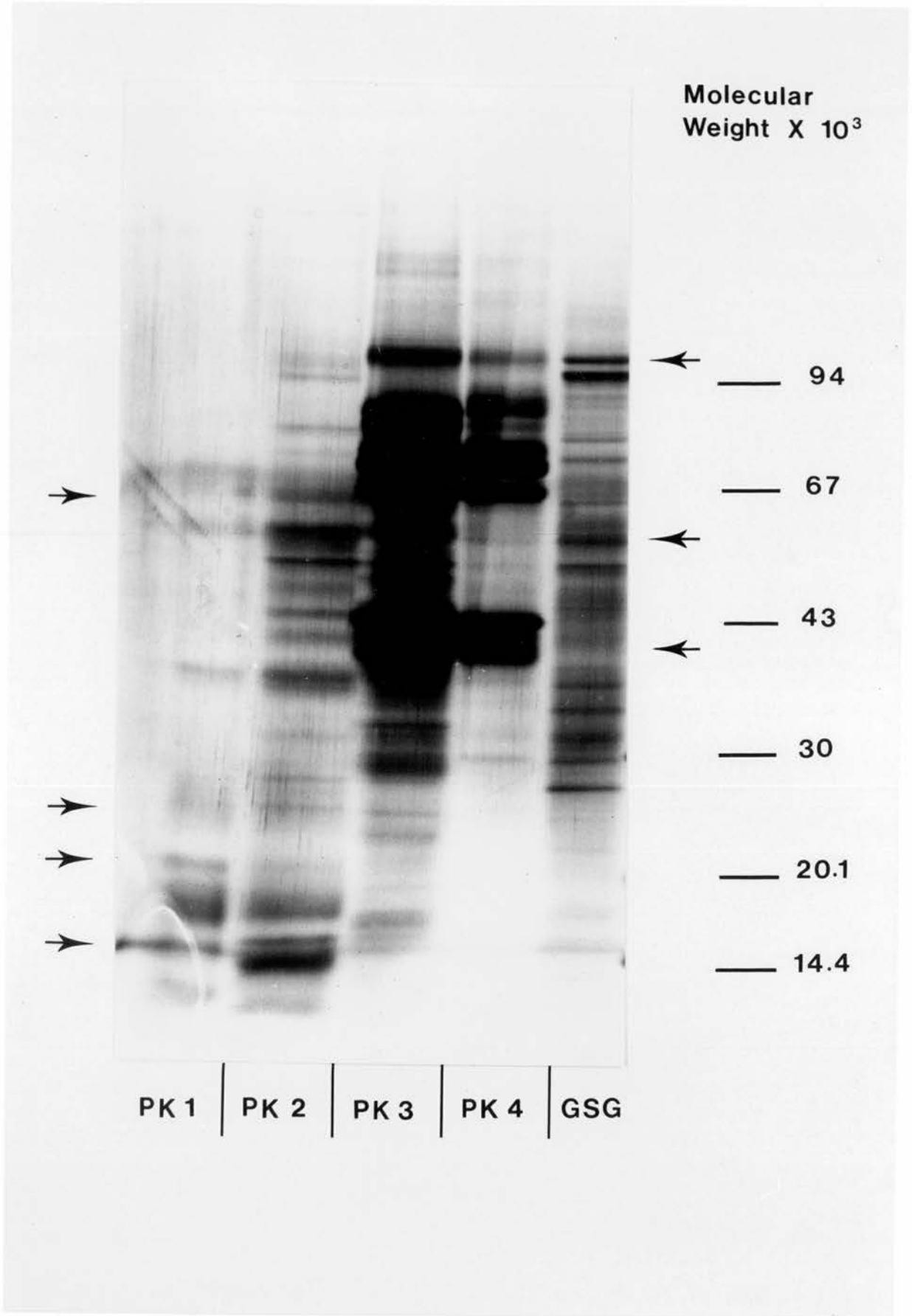


Figure 4.2. Comparative diagram of the changes in absorbance observed in the eluates of the DE-52 chromatographic separations of salt fractioned salt fractioned Rhipicephalus appendiculatus larval extracts. The lines indicate the relative changes in the protein content of the eluate of the columns. Results on the chromatographic separation of five different materials are illustrated. The separations used either stepwise changes in the molarity of sodium chloride in the eluant buffer (solid lines and changes of buffer indicated by arrows) or a linear gradient of sodium chloride from 0 to 300 mM in the eluant buffer (dashed lines). The code for each material is indicated at the right of each diagram, using the same nomenclature as in table 4.1.

Figure 4.3. SDS-PAGE protein profiles of the peaks of protein eluted during the chromatographic separation of Rhipicephalus appendiculatus larval extracts. Gels used a gradient concentration of acrylamide from 7% to 20%. PK1, PK2, PK3 and PK4 identify the different peaks of protein, GSG = salivary gland homogenate of females used for reference. The numbers indicate the migration exhibited by markers of respective molecular weight. Protein bands in the gel were visualised using the silver staining technique of Morrissey (1981). The arrows at the left indicate protein bands in PK1 and PK2 refered in the text. Arrows at the right, indicate the protein bands in PK3 and PK4 described in the text.


Molecular
Weight X 10³
94
67
43
30
20.1
14.4
PK 1
PK 2
PK 3
PK 4
GSG

(3.4.3).

In PK1 there were typically 3 bands of protein with molecular weights of 15, 20 and 22 kilodaltons (kD). PK2 contained these three proteins plus 10 protein bands of intermediate molecular weights (from 25 to 62 kD). Fractions PK3 and PK4 contained more abundant proteins bands than PK1 or PK2, and they were clearly separated into two groups, group one had 3 - 4 major bands around 40 kD and group two had 8 major bands ranging from 55 to 99.5 kD. Another fainter group of at least three bands was observed between 40 and 55 kD on Pk3 and PK4, but it was more intense in PK3.

Chromatographic separation of the proteins contained in GSG was also attempted. The results of these observations are summarized in figure 4.4. Repeatibility of the procedure, using step changes of salt concentration of the eluant, was low. This lack of repeatibility could be attributed to the low protein concentration found in the eluant of the columns, but in spite of this a clear separation of proteins was obtained (figure 4.5). GSG consisted of a complex mixture of proteins, containing at least 40 major bands, with molecular weights ranging from 10 to 97 kD. PK1 displayed 2 very faint bands of 40 and 43 kD. PK2 and PK3 were very similar and consisted of three bands (55, 62 and 70 kD). All these bands were found with more intensity in PK4, where 3 additional bands were detected (30, 38 and 72 kD).

These observations indicate that much larger amounts of starting material were required for the practical fractionation of these homogenates by anion-exchange chromatography. This procedure was therefore considered impractical because of the amount of labour required for dissecting the salivary glands from the tick.

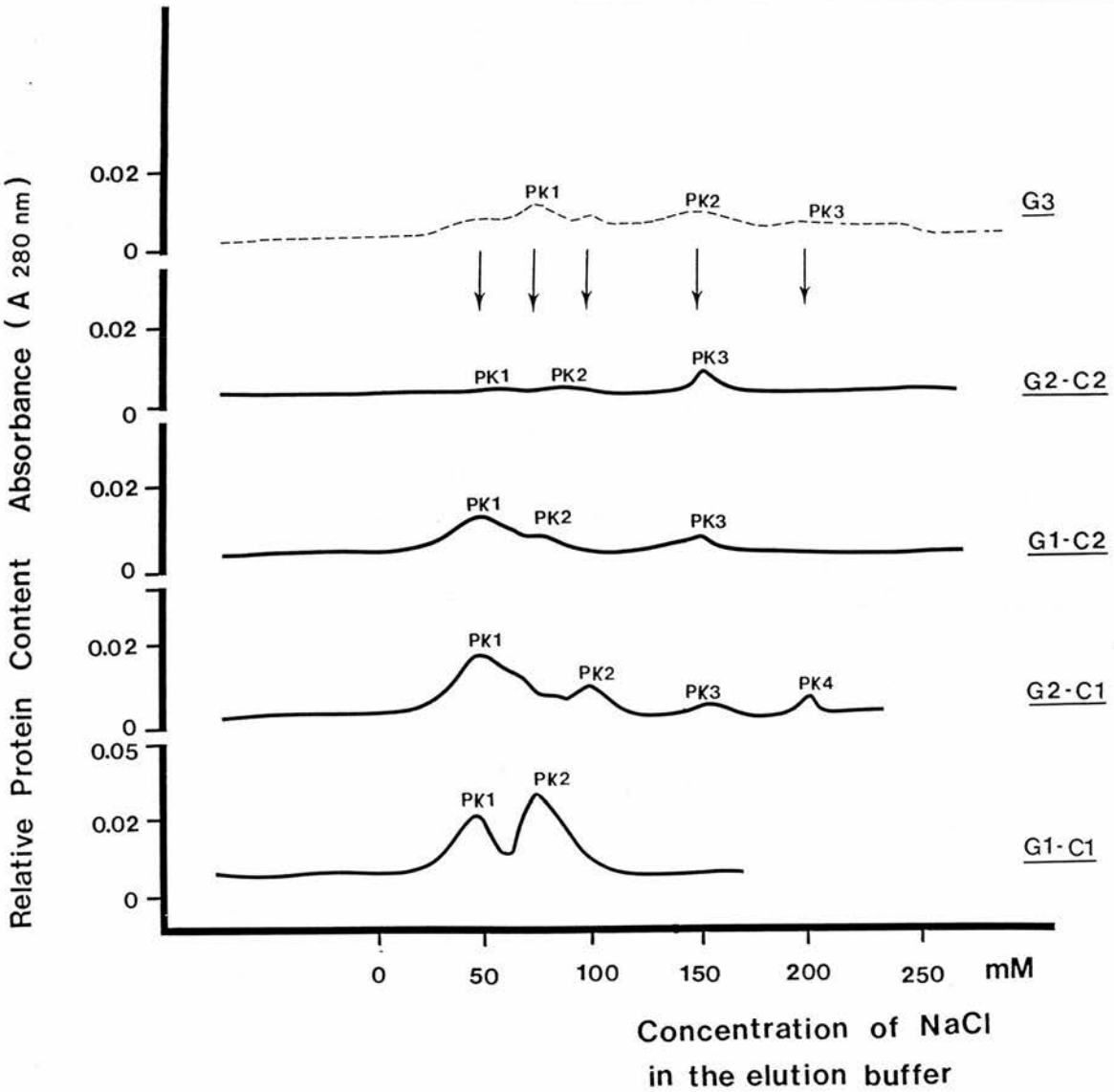
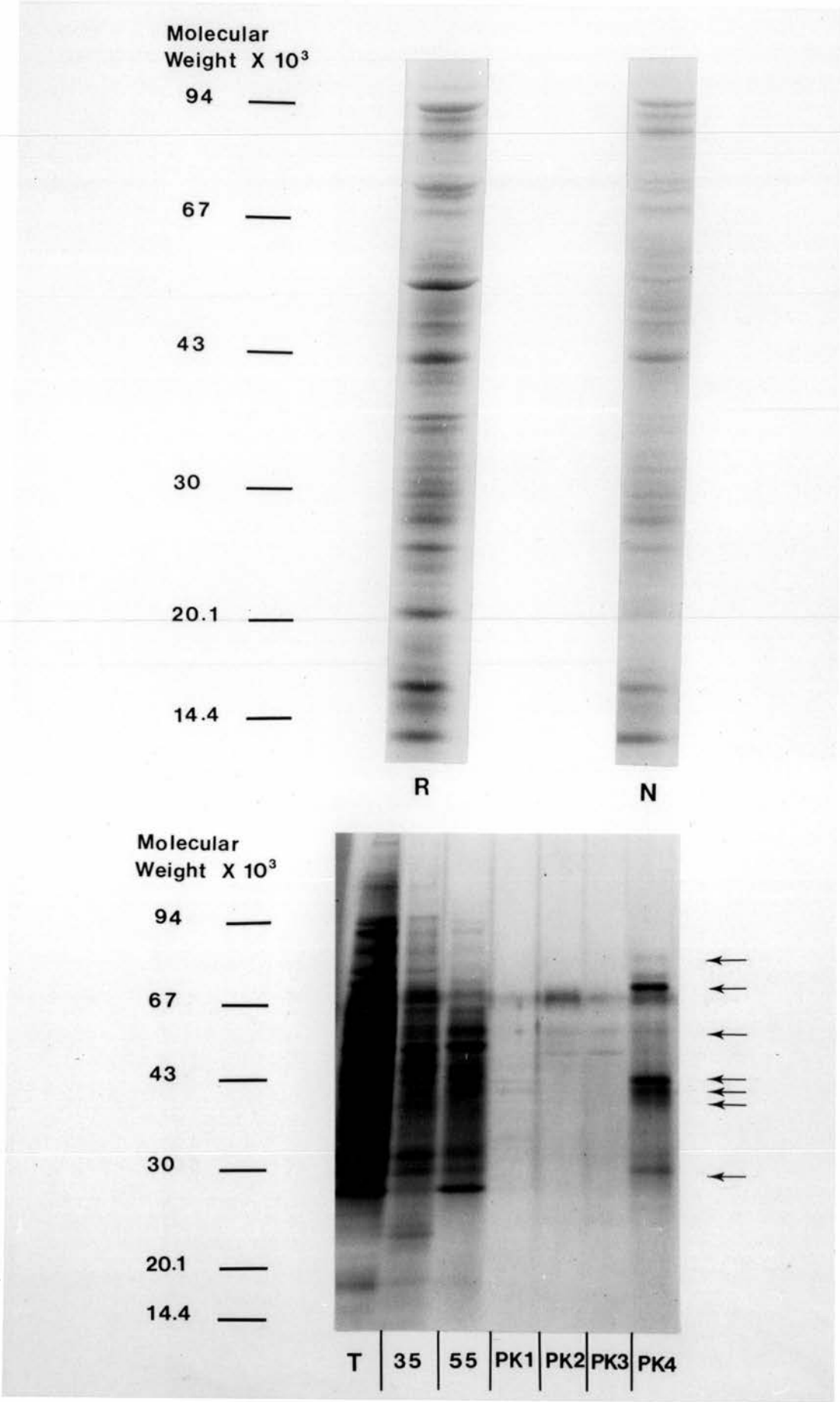


Figure 4.4. Schematic representation of the protein content found in the eluant of DE-52 columns to demonstrate the repeatibility during fractionation of different salivary gland homogenates from Rhipicephalus appendiculatus females. Materials loaded into the columns included homogenates fractionated at concentrations of saturation of ammonium sulphate of 35% (-C1) or 55% (-C2) as well as crude material (G3). The separations were conducted using either stepwise changes in the molarity of sodium chloride in the solvent (solid lines and the changes of buffers indicated by arrows), or a linear gradient of sodium chloride from 0 to 300 mM in the eluant buffer (dashed line).

Figure 4.5. Protein profile on SDS-PAGE (gradient 7 - 20% acrylamide) of salivary gland homogenates from Rhipicephalus appendiculatus females at various stages during fractionation of the extracts by salt precipitation and by anion exchange chromatography. The top lanes illustrate the protein patterns of extracts prepared each with 30 pairs of salivary glands dissected from ticks fed on rabbits either naive (N) or resistant (R) to the tick. (Proteins stained with coomassie blue stain). The bottom lanes indicate the separation of proteins observed on different steps of fractionation of a salivary gland homogenate T = total homogenate, 35-55 = proteins precipitated at corresponding concentrations of ammonium sulphate, PK1, PK2, PK3 and PK4 = materials fractionated by chromatography of the 55 extract. (Proteins were stained with silver stain using the method of Morrissey (1981)). The numbers indicate the migration exhibited by calibration proteins of corresponding molecular weight. The arrows correspond to bands described in the text.


Molecular
Weight X 10³
94
67
43
30
20.1
14.4
R
N
Molecular
Weight X 10³
94
67
43
30
20.1
14.4
T
35
55
PK1
PK2
PK3
PK4

In an attempt to improve the protein concentration of GSG, the protein profiles of salivary homogenates prepared with identical numbers of salivary glands obtained from ticks either fed on susceptible or on resistant rabbits were compared (figure 4.5). A similar profile of proteins was found in both extracts, but higher concentrations of proteins were detected in ticks fed on resistant rabbits, this material was therefore used for the isolation of antigens. A crude extract of these glands was chromatographed (G3), but still the protein concentration of the eluant was too low to make the procedure practical.

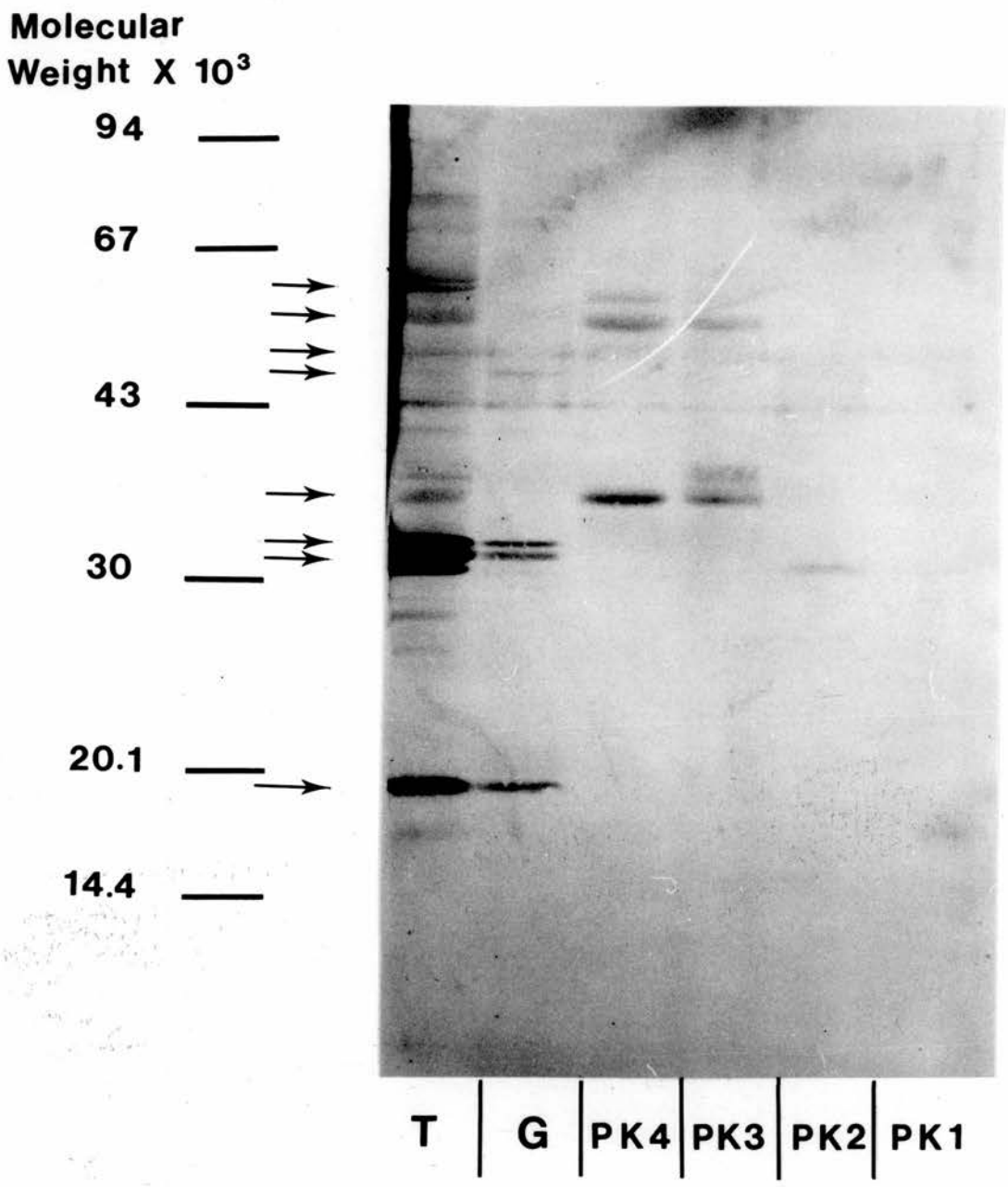
Antigenic proteins contained either in unfractionated GSG or in total and in purified materials from larvae, were identified by Western blotting using sera from an immune rabbit (3.6.3). At least 15 antigens were identified in the total larval extract (figure 4.6), of which five showed identity with molecules recognized on the salivary gland extract (approximate molecular weight: 19, 33, 34, 48 and 52 kD). Three antigens with molecular weights 38, 59 and 64 kD were also recognized in fractions PK3 and PK4. The band of 33 kD was also identified in material PK2.

4.3.2.- Fractionation of B. microplus larval extracts.

The description of the work using B. microplus larvae is divided in two sections: chromatographic separations performed at CTVM using DE-52 as anion-exchanger and separations performed at LIMV using DEAE-Sephadex A-50.

4.3.2.1.- Chromatographic separations performed at CTVM

Figure 4.6. Pattern of antigens contained in different materials extracted from the tick Rhipicephalus appendiculatus as identified by Western blotting. Materials were separated by SDS-PAGE on 7-20% gradient acrylamide gel slabs, electroblotted, incubated with sera of a immune rabbit and developed with a peroxidase conjugated goat anti-rabbit immunoglobulin G (GAR-IgG). T = Total larval extract, G = salivary gland homogenate. PK1, PK2, PK3 and PK4 = materials purified from larval extracts by chromatography. The arrows indicate bands described in the text.


Molecular
Weight X 10³
94
67
43
30
20.1
14.4
T
G
PK4
PK3
PK2
PK1

A summary of the fractionation of larval proteins from B. microplus attained at CTVM is presented in figure 4.7. Three protein peaks were consistently observed (Peak 1 at 50 mM sodium chloride, Peak 2 at 75 to 100 mM sodium chloride and Peak 3 at 150 mM sodium chloride). Peak 4 was observed when a higher salt concentration (350 mM) was used as eluant. The patterns of separation of proteins are illustrated on figure 4.8. Single faint bands of low molecular weight were observed in PK1 (18.8 kD) and PK2 (17.5 kD) whilst PK3 contained two groups of proteins in a manner similar to that described for R. appendiculatus extracts. One of these groups consisted of four bands centred on 43 kD and the other group of 7 bands of protein between 68 - 106 kD. Corresponding fractions obtained in various chromatographic separations were pooled and freeze dried to be transported to Colombia for their use in field trials (chapters six and seven).

Western blotting was used to study the antigenicity of the B. microplus materials obtained during the various steps in the purification procedures conducted at Edinburgh. For this sera from immune animals (a pool of sera from the five cattle more reactive on the ELISA test described in chapter three) was used. A salivary gland homogenate from B. microplus females was used as reference material (figure 4.9).

The immune sera recognized antigens only in the total larval extract (result not shown) and the products of the preliminary salt fractionation. In materials precipitated at either 35% or 55% saturation of ammonium sulphate, two antigens, molecular weight 37 and 39 kD were identified. The 39 kD band showed identity with an antigen recognized on the salivary gland homogenate. No antigens

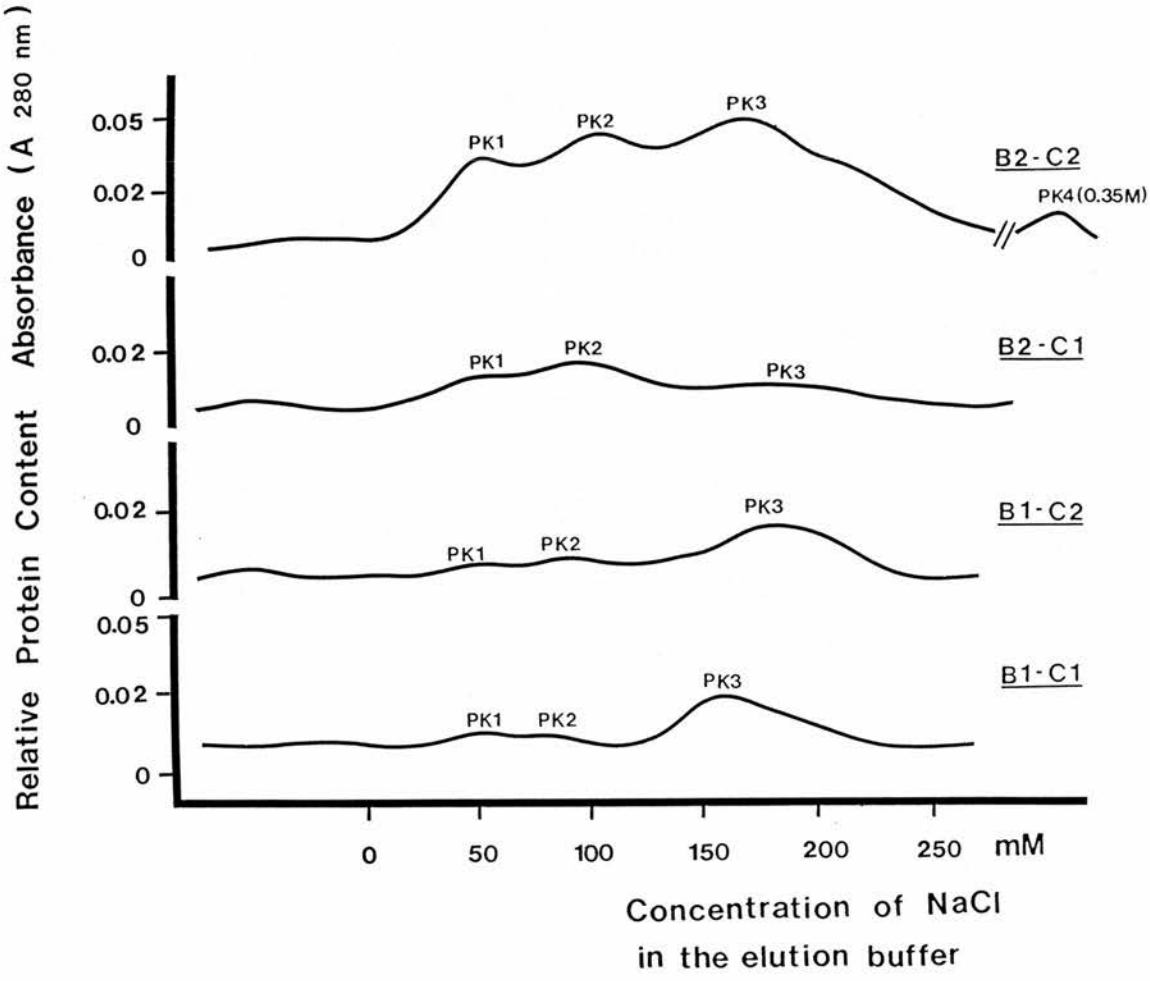


Figure 4.7. Anion-exchange chromatography of Boophilus microplus larval extracts using DE-52. The lines indicate the changes in protein content of the eluate of the columns after chromatography of extracts precipitated at different saturation levels of ammonium sulphate (-C1 = 35%, -C2 = 55%). All columns used a linear gradient of molarity of sodium chloride (0-300 mM) for the elution of proteins.

Figure 4.8. Proteins contained in larval extracts of Boophilus microplus ticks at different stages of fractionation, as displayed by electrophoresis on SDS-Polyacrylamide gels (gradient 7-20% concentration total acrylamide). BOOM identifies the total original extract. 35 and 55 identify proteins precipitated by the corresponding percentages of saturation of ammonium sulphate. PK1, PK2 and Pk3 identify peaks of protein eluted after anion-exchange chromatography of the 55 extract. RAPP identify a total larval extract of Rhipicephalus appendiculatus ticks used for reference. The proteins were visualised using the silver stain method of Morrissey (1981)

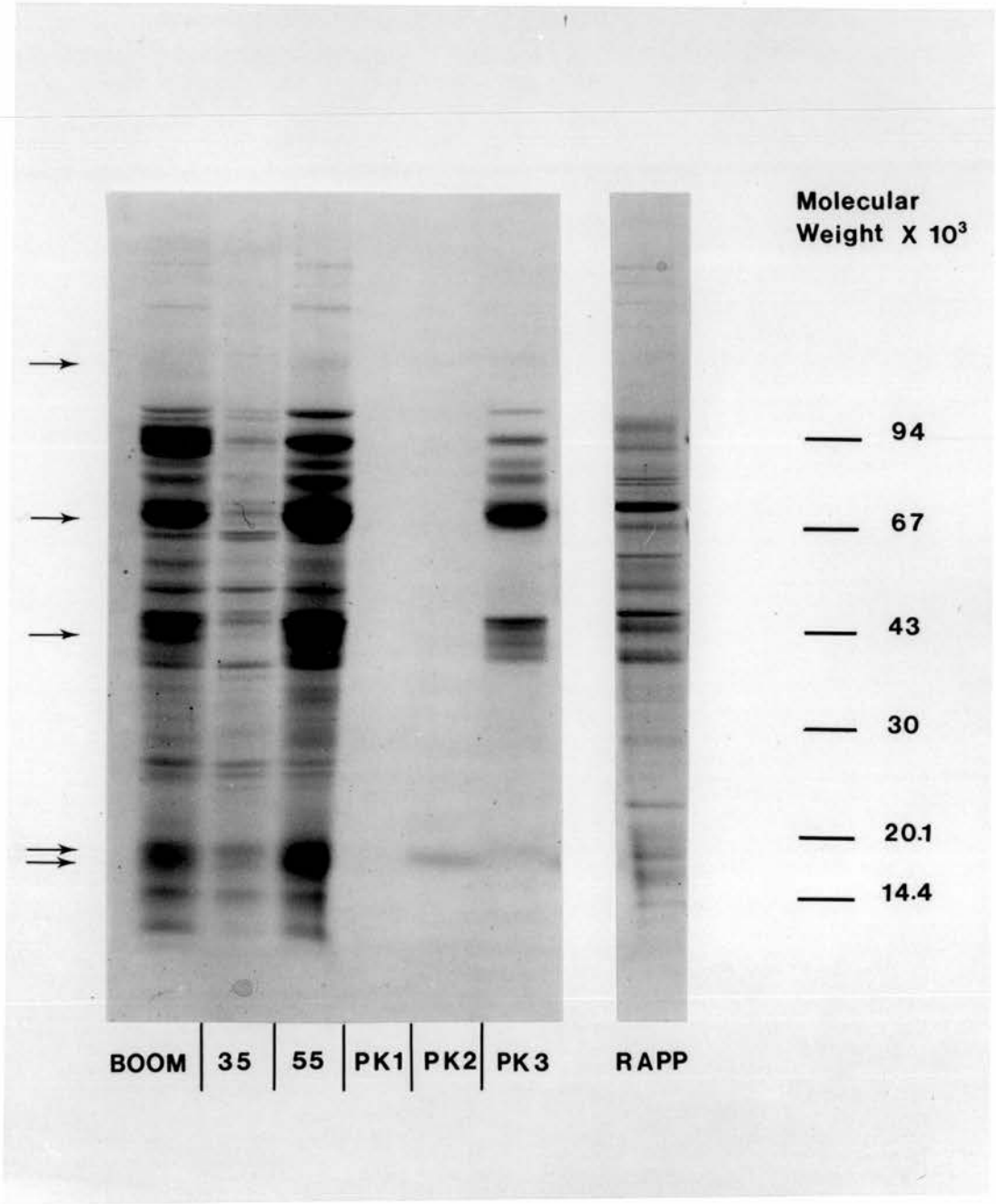
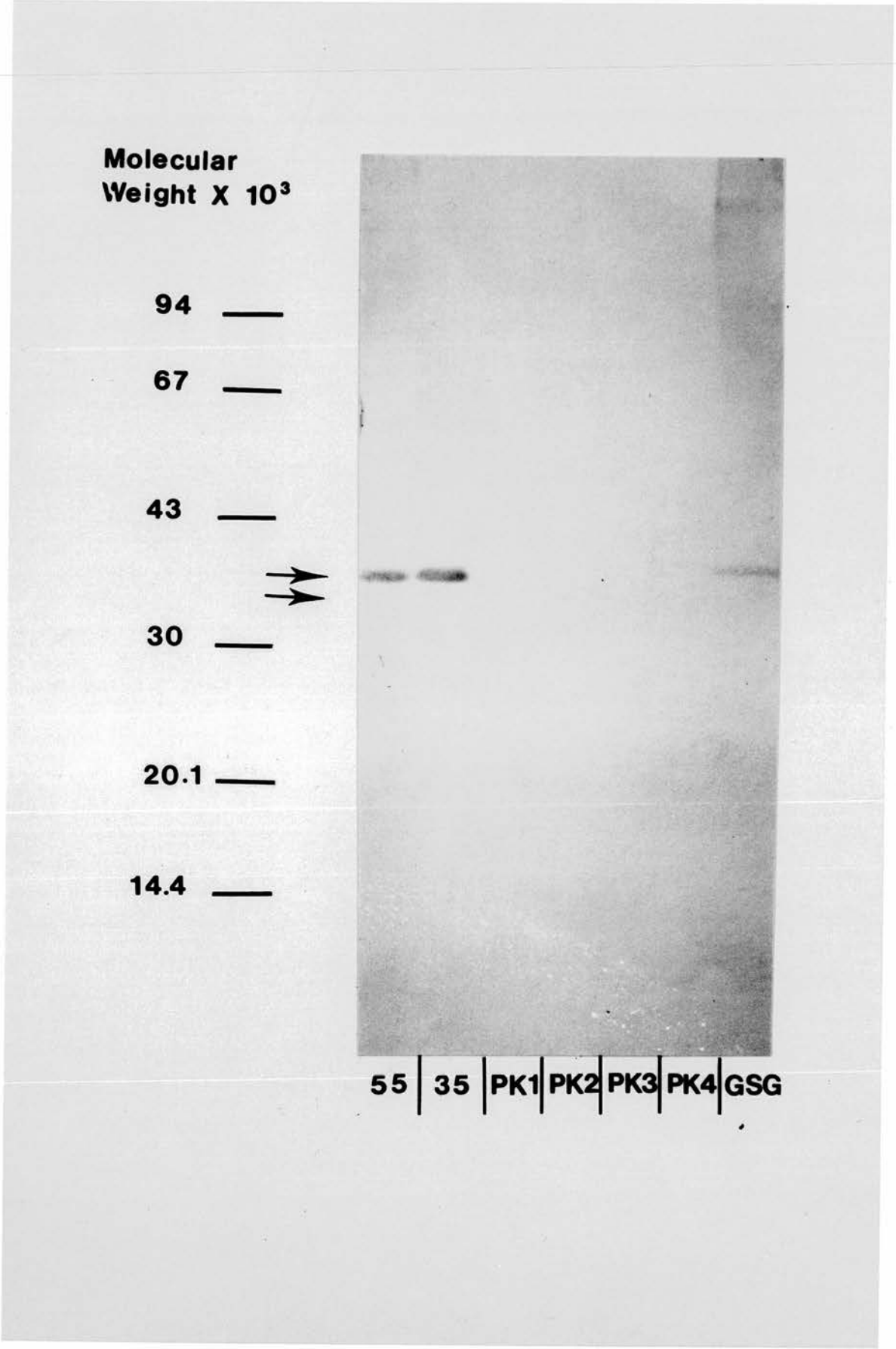

Molecular
Weight X 10^3
94
67
43
30
20.1
14.4
BOOM
35
55
PK1
PK2
PK3
RAPP

Figure 4.9. Proteins recognized by sera of immune calves on different protein mixtures extracted from the tick Boophilus microplus. After separation by SDS-PAGE (gel slabs on an acrylamide gradient from 7-20%), proteins were transferred to nitrocellulose paper, incubated with the immune sera and developed with peroxidase conjugated Goat anti-bovine immunoglobulin G (GAB-IgG). 55 and 35 respectively identify larval derived proteins precipitated by ammonium sulphate at concentrations of saturation of 55% and 35%. PK1, PK2, PK3 and PK4 are proteins fractionated from the 55 extract by anion-exchange chromatography on DE-52. GSG is a salivary gland homogenate from female ticks. The arrows point to bands described in the text.


Molecular
Weight X 10^3
94
67
43
30
20.1
14.4
55
35
PK1
PK2
PK3
PK4
GSG

were detected in PK1, PK2 or PK3.

4.3.2.2.- Chromatographic separations performed at LIMV.

The same techniques developed at the CTVM were implemented using the facilities available at LIMV and DEAE-Sephadex A-50 as anion-exchanger. A clearly different elution pattern of proteins was observed when the 35% ASP or 65% ASP fractions were separated.

Figure 4.10 illustrates the results obtained on the separation of the 65% ASP fractions and figure 4.11 illustrates the separation of the 35% fractions. Higher yields of protein were produced by the 65% precipitates. The protein concentration of the eluate displayed a common pattern in accordance with the molarity of sodium chloride used to elute the proteins from the anion-exchanger.

The procedure showed good repeatibility, about 70 fractions (8-9 mls each) were produced on each separation and they were usually pooled as six major peaks, except on chromatogram L2-65 where seven peaks were separated. The allocation of a fraction to a peak was made on the basis of visual judgement according to the shape of the curve and to the calculated salt molarity of the eluate. Peaks were identified as P1, P2, P3, P4, P5, P6 and D2 (figures 4.10 and 4.11).

Pooled peaks were concentrated to about 1/10 of their original volume (the original volume of each peak varied from 50 to 150 ml) and divided into equal fractions, (0.5 - 1.0 ml). The protein content was estimated and the fractions were frozen at -20°C (the space in the deep freezer was reserved for these purified materials). Results of these procedures are shown in table 4.3.

Concentration on polyethylene glycol was used in the initial

Figure 4.10. Anion-exchange chromatography on DEAE-Sephadex A-50 of Boophilus microplus larval extracts partially fractionated by precipitation between 35% to 65% saturation on ammonium sulphate (65% ASP). A 600 ml linear gradient of sodium chloride from 0 to 500 mM on 50mM Tris-HCl buffer, pH 8.7 was used as eluant. Results on the chromatographic separations of five different materials are presented. L2 to L10 indicate the larval extract used for each one. P1, P2, P3, P4, P5, P6 and D2 show the fractions pooled as peaks for subsequent analysis.

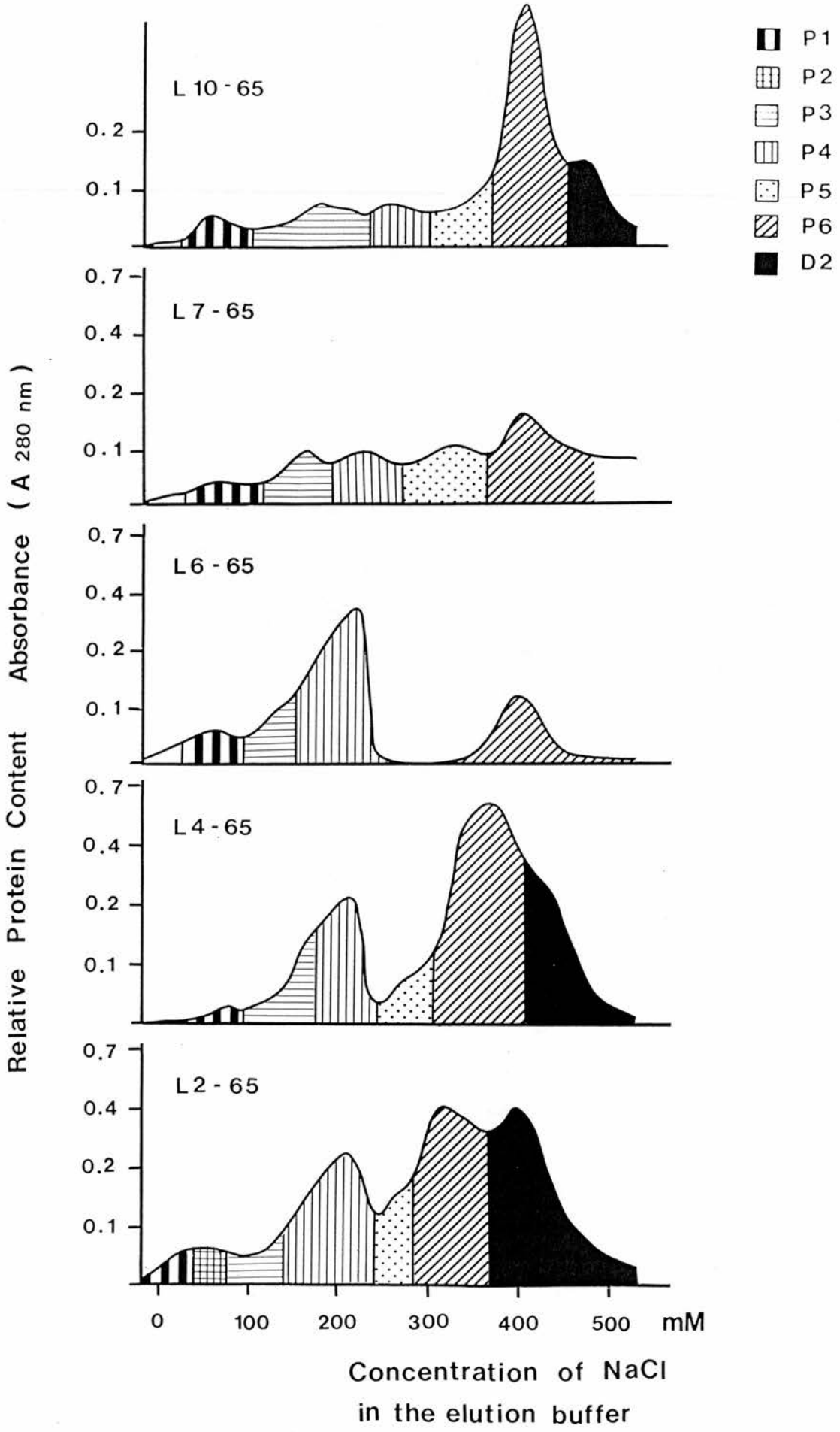

P1
P2
P3
P4
P5
P6
D2
L 10 - 65
L 7 - 65
L 6 - 65
L 4 - 65
L 2 - 65
0.2
0.1
0.7
0.4
0.2
0.1
0.7
0.4
0.2
0.1
0.7
0.4
0.2
0.1
0.7
0.4
0.2
0.1
Relative Protein Content Absorbance (A 280 nm)
0
100
200
300
400
500
mM
Concentration of NaCl
in the elution buffer

Figure 4.11. Chromatographic separation on DEAE-Sephadex A-50 of four different Boophilus microplus larval extracts (L3 to L10) subjected to salt fractionation at 35% saturation of ammonium sulphate. A 600 mls linear gradient (0-500 mM) of sodium chloride on Tris-HCl buffer (50 mM, pH 8.7) was used as eluant. P1, P3, P4, P5, P6, and D2 indicate the fractions pooled as peaks for subsequent analysis.

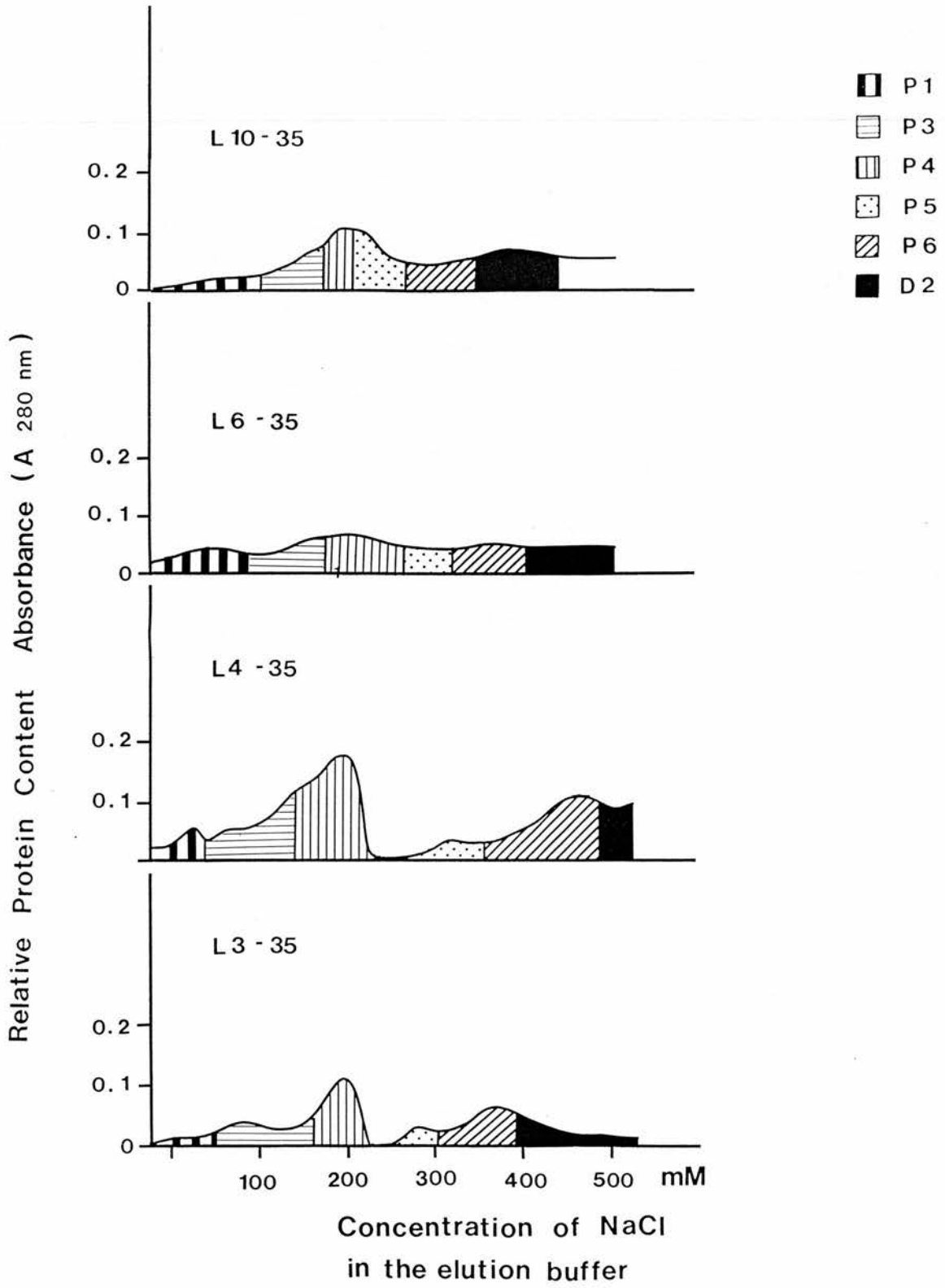

P1
P3
P4
P5
P6
D2
L 10 - 35
L 6 - 35
L 4 - 35
L 3 - 35
0.2
0.1
0
Relative Protein Content Absorbance (A 280 nm)
100
200
300
400
500
mM
Concentration of NaCl
in the elution buffer

Table 4.3. Protein content of the eluted peaks (after being concentrated) when Boophilus microplus larval extracts were separated using anion-exchange chromatography on DEAE-Sephadex A-50

		COLUMN FRACTIONS						
Columns	Date	P1	P2	P3	P4	P5	P6	D2
		Protein concentration (micrograms/ ml) *						
L2-65	06-87	60	103	N.D.	274	N.D.	1800	2057
L4-65 (1)	08-87	10	-	26	193	610	905	415
L4-65 (2)	12-87	17	-	38	475	750	3800	2865
L4-35	12-87	11	-	20	69	N.D.	505	1475
L3-35	01-88	7	-	N.D.	N.D.	80	350	690
L6-65	02-88	9	-	19	30	38	1120	-
L7-65	04-88	48	-	47	150	250	550	-
L6-35	05-88	16	-	21	16	17	13	13
L10-65	08-88	43	-	40	80	130	2400	270
L10-35	08-88	9	-	21	80	60	11	80

N.D. No material available for test.

* = Estimated using a coomassie blue G-250 binding assay. (Bradford, 1976; Bearden, 1978). See chapter three (3.2).

chromatographic separations (columns L2-65 and L4-65{1}), but there were difficulties of detecting protein accurately in some materials (P1, P3). It was thought that this was due to low molecular weight proteins being lost during concentration (molecular weight cut off of the dialysis bags 12,000 - 14,000). This caused a change to the use of ultrafiltration membranes (5,000 M.W. cut off) but no improvements in the recovery of protein after concentration were observed with their use.

By this time (November 1987), field results were available that indicated allergenic activity of these materials when skin tested in spite of their low protein concentration (8.3.1). This required a more sensitive technique to measure the amount of protein contained on those materials.

A technique to measure protein in the range of 0.5 to 50 µg/ml was then standardized (Bearden, 1978) and this analysis conducted again on the preserved materials when available. As concentration by ultracentrifugation proved to be laborious, it was decided to remain with the polyethylene glycol method of protein concentration.

Materials from columns L2-65 and L4-65 were used in preliminary evaluations in cattle exposed to the tick and all showed capability to induce immediate hypersensitivity reactions (8.2.3). A combination of materials from columns L4-65, L6-65 and L7-65 were used for the final experiment of evaluation of two methods to measure resistance to the tick (9.2.4).

During the final period of study at Edinburgh, the pattern of proteins contained in the different isolated materials were studied by SDS-PAGE. All the different isolates corresponding to the same peak were electrophoretically separated in the same gel. The

proteins were revealed by both the coomassie blue stain and by the silver stain (3.4.3), and the pattern of bands present on each slab was recorded.

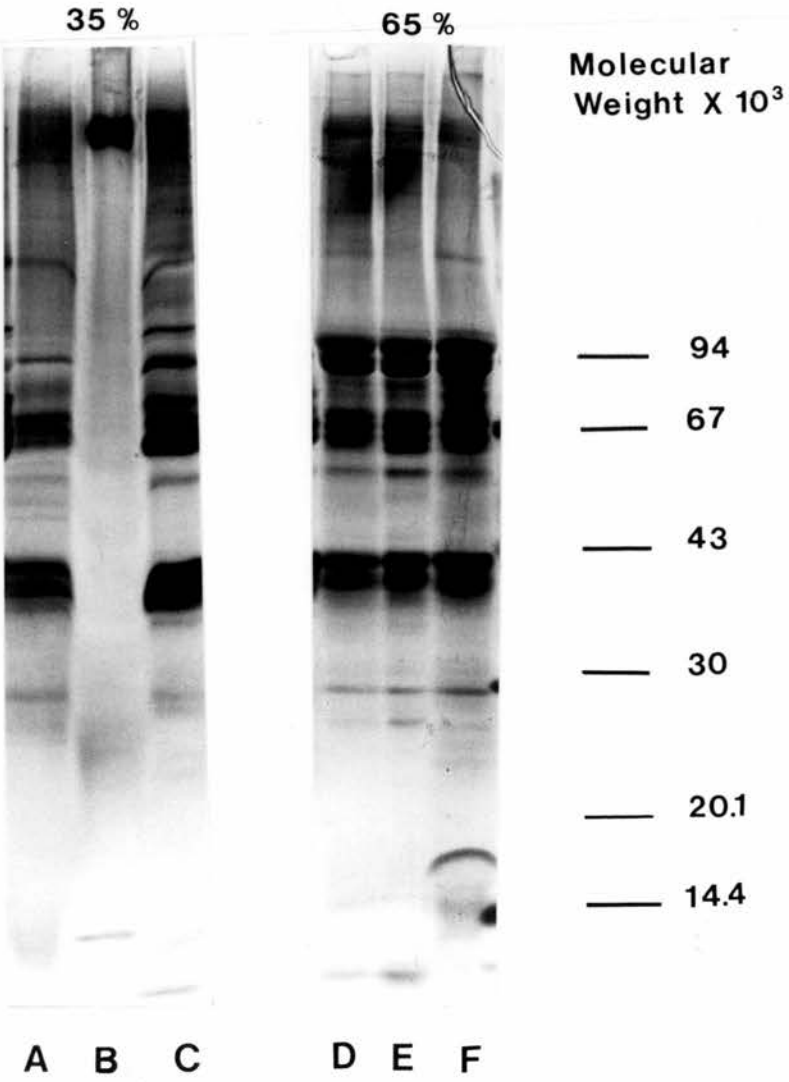
Indices of similarity between the different isolates of each peak were calculated. Figure 4.12 exemplifies the calculation of a dendrogram based on the indices of similarity between different isolates of the material D2. Figure 4.13 shows the dendrograms for materials P1, P3 and P4. Figure 4.14 illustrate the dendrograms constructed with the indices of similarity between different isolates of materials P5, P6 and D2.

For each peak, the similarity of materials obtained on different chromatographies was generally over 40%. On P1, P4, P6 and D2 low similarity was observed for one or two materials obtained after chromatography of the 35% ASP fractions. These low indices of similarity appeared to be caused by the difficulty of detecting faint bands on the gels.

SDS-PAGE analysis was conducted on a pool of each peak and results are displayed on figure 4.15. Materials P1, P3 and P4 were characterized by having faint bands of proteins of low molecular weight arranged by their size in two groups, one between 14 and 22 kD and the second between 28 and 32 kD. The indices of similarity between these fractions were fairly low (P1/P3 = 33%, P1/P4 = 24%, P3/P4 = 48%) indicating good degree of separation of proteins during chromatography.

Materials P5, P6 and D2 contained three major groups of protein. Group one contained proteins with molecular weights between 40 to 48 kD, group two between 63 to 77 kD and group three of

Figure 4.12. Electrophoretic pattern on polyacrylamide gels (gradient 7-20%) of various isolates of the material D2, extracted from Boophilus microplus larvae by anion-exchange chromatography using larval extracts precipitated at different concentrations of saturation of ammonium sulphate (35-65%). The dendrogram illustrates the index of similarity (based on the proportion of protein bands that are common to two gels slabs being compared) calculated after recording the bands visualized either by the coomassie blue or by the more sensitive silver stain. The location of each material on the dendrogram is indicated by the letter at the bottom of each slab. The code that follows each letter on the dendrogram identifies the number of the original larval extract (L3-L10) and the concentration of ammonium sulphate on which it was precipitated (35-65). The migration exhibited by calibration proteins of known molecular weight is also indicated.


35 %
65 %
Molecular
Weight X 10^3
94
67
43
30
20.1
14.4
A B C D E F

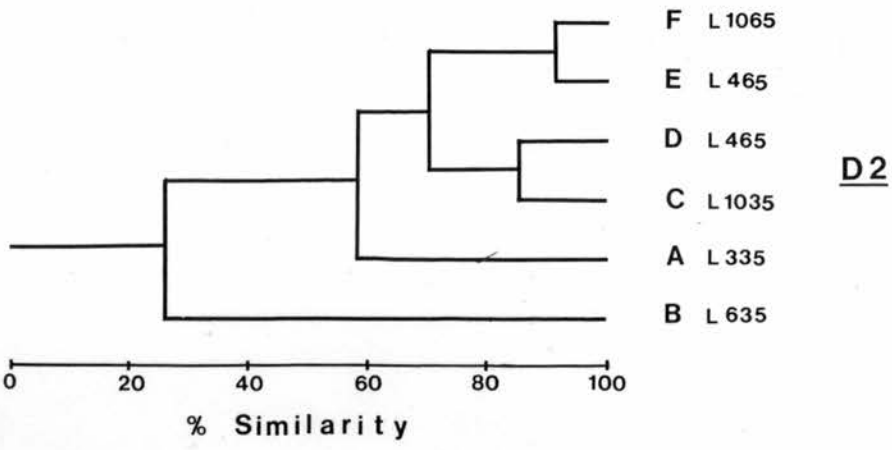
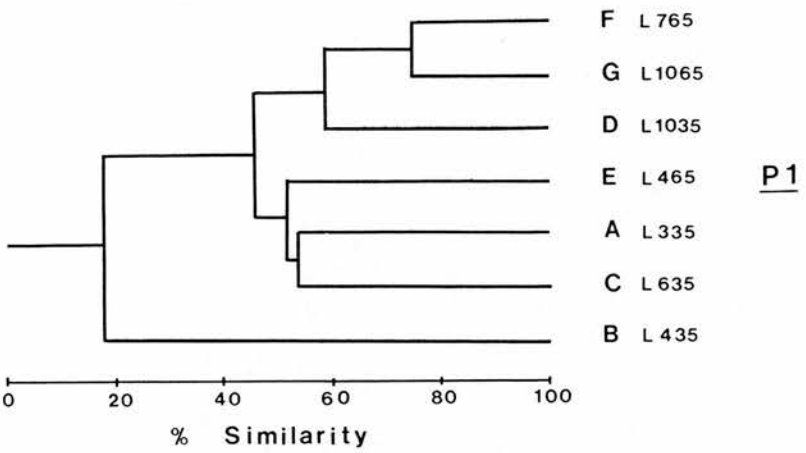
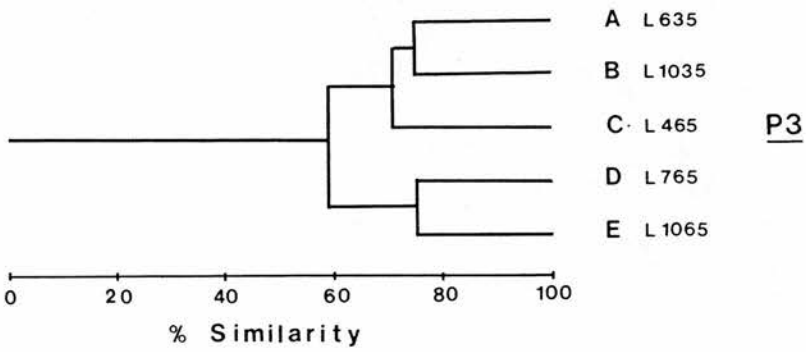

F L 1065
E L 465
D L 465
C L 1035
A L 335
B L 635
D2
0 20 40 60 80 100
% Similarity

Figure 4.13. Dendrograms to illustrate the indices of similarity between various isolates of materials purified from larval extracts of the tick Boophilus microplus using chromatographic methods. Results for the materials **P1**, **P3** and **P4** are presented. For each material a separate dendrogram was constructed. Each index of similarity was calculated based on the protein electrophoretic pattern on SDS-PAGE of each isolate, by comparing the proportion of protein bands that were common to two gels slabs being examined. The bands of protein in the gel slabs were recorded after visualization by staining the gel either, with coomassie blue or with a more sensitive silver stain (Morrissey, 1981). These isolates were obtained after chromatography of larval extracts (L3-L10) previously fractionated by precipitation on different concentrations of of ammonium sulphate (35-65% saturation). The location of each material on the dendrogram is indicated by a different letter. The code that follows each letter identifies the number of the original larval extract (L3-L10) and the concentration of ammonium sulphate on which it was precipitated (35-65).


F L 765
G L1065
D L1035
E L 465
A L 335
C L 635
B L 435
0
20
40
60
80
100
% Similarity

P1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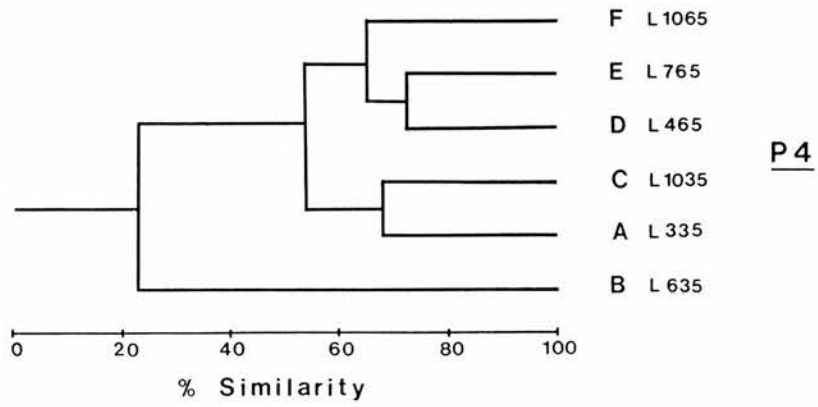
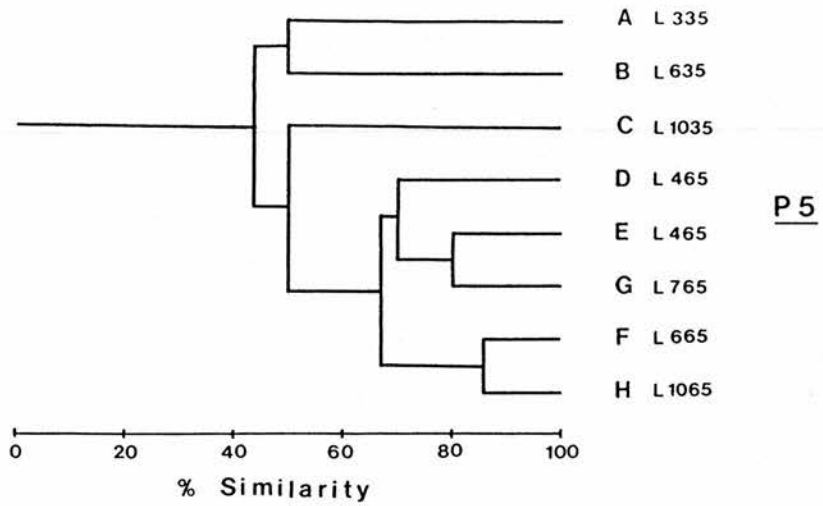
B L1035
C L 465
D L 765
E L1065
0
20
40
60
80
100
% Similarity

P3

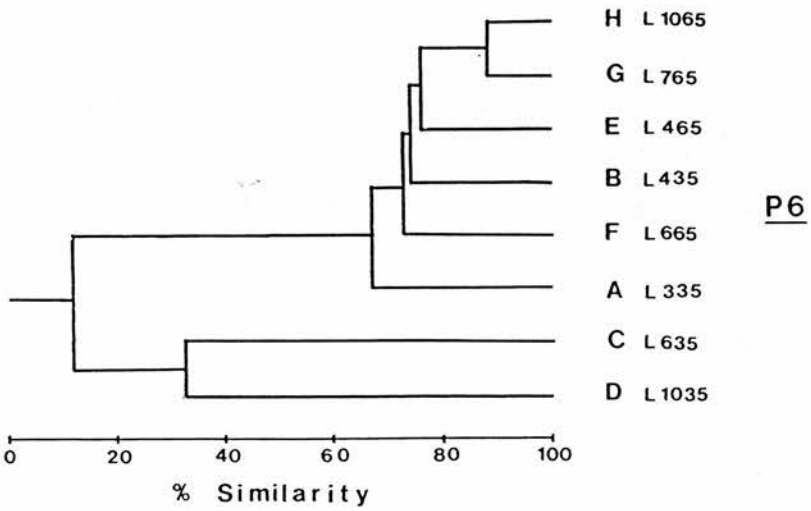

F L1065
E L 765
D L 465
C L1035
A L 335
B L 635
0
20
40
60
80
100
% Similarity

P4

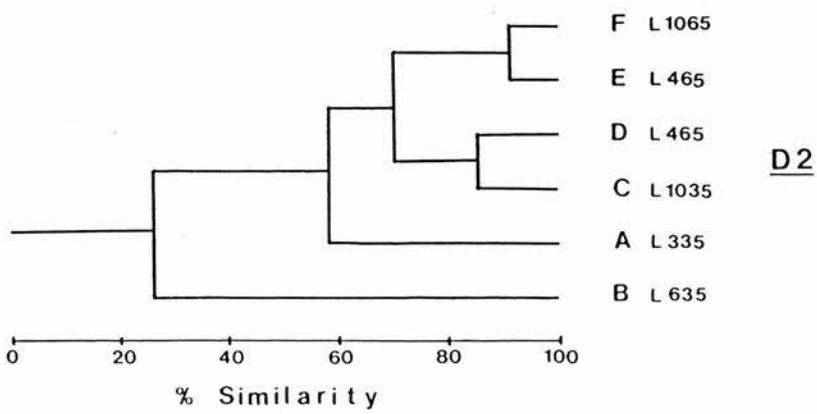
Figure 4.14. Dendrograms to illustrate the indices of similarity of various isolates of materials purified from larval extracts of the tick Boophilus microplus. Results for the materials **P5**, **P6** and **D2** are presented (For each material a separate dendrogram was constructed). The indices of similarity were calculated as explained on figure 4.13. The bands of protein in the gel slabs were recorded after being visualized by staining the gel either with coomassie blue or with a more sensitive silver stain (Morrissey, 1981). These isolates were obtained after chromatography of larval extracts (L3-L10) previously fractionated by precipitation on different concentrations of of ammonium sulphate (35-65% saturation). The location of each material on the dendrogram is indicated by a different letter. The code that follows each letter identifies the number of the original larval extract (L3-L10) and the concentration of ammonium sulphate on which it was precipitated (35-65).


A L 335
B L 635
C L 1035
D L 465
E L 465
G L 765
F L 665
H L 1065
0
20
40
60
80
100
% Similarity

P5

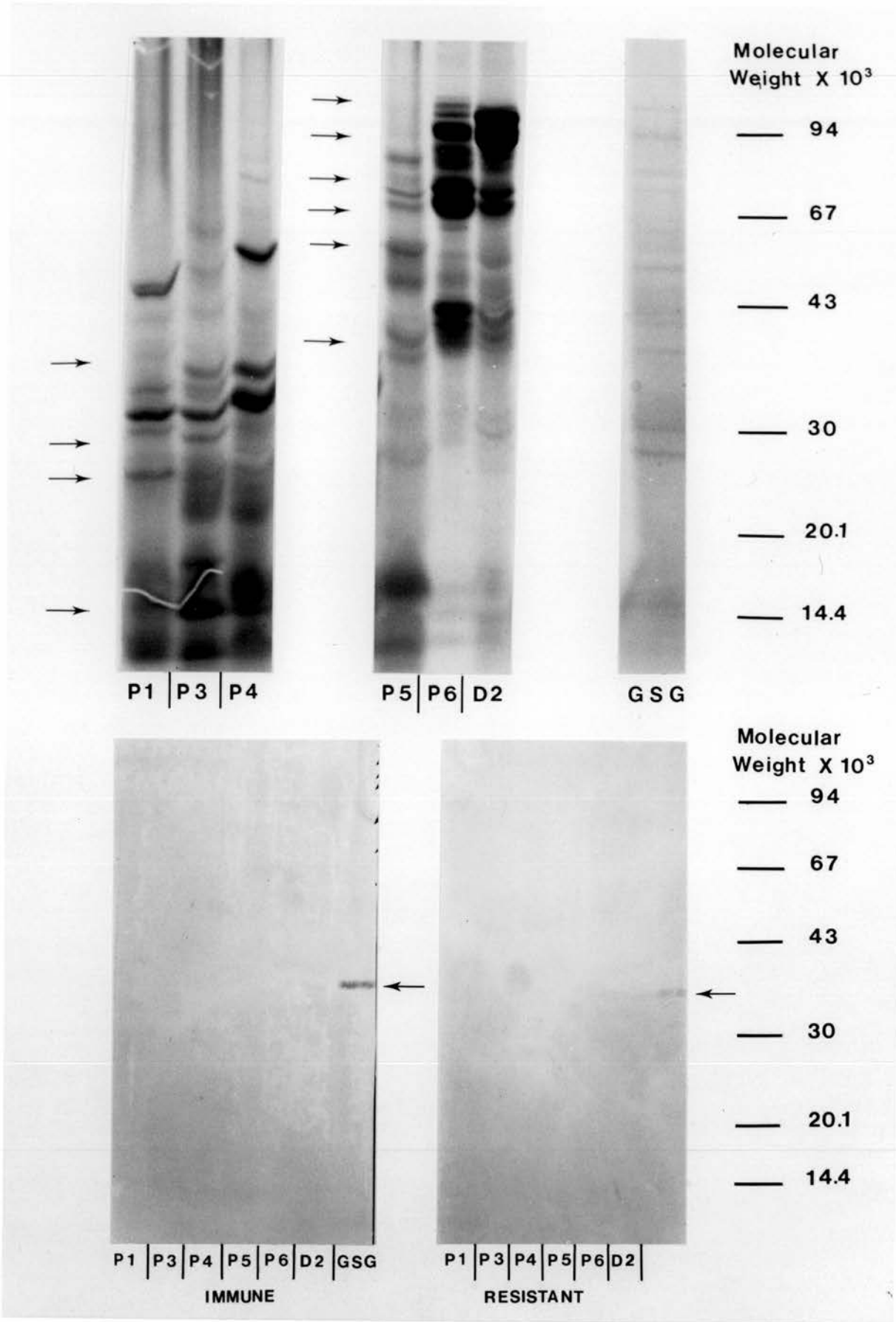

H L 1065
G L 765
E L 465
B L 435
F L 665
A L 335
C L 635
D L 1035
0
20
40
60
80
100
% Similarity

P6


F L 1065
E L 465
D L 465
C L 1035
A L 335
B L 635
0
20
40
60
80
100
% Similarity

D2

Figure 4.15. SDS-PAGE protein profile of different materials purified from Boophilus microplus larvae by anion-exchange chromatography on DEAE-Sephadex A-50 (P1, P3, P4, P5, P6, D2) and of a homogenate of salivary glands extracted from adult ticks (GSG). Electrophoresis was conducted on a gel with a gradient concentration of acrylamide from 7 to 20%, and proteins were visualized using a coomassie blue stain (Top plate). Proteins identically separated were electroblotted to nitrocellulose paper and immunoreactive molecules were identified (bottom plate) using a peroxidase conjugated goat anti-bovine immunoglobulin G (GAB-IgG) after incubating the transferred proteins with sera from calves either immune or resistant to the tick (see text for explanations). Materials of larval origin consisted of a pool of materials resulting from different chromatographic separations. Migration exhibited by proteins of known molecular weight is indicated. The arrows point to bands described in the text.


Molecular Weight X 10^3
94
67
43
30
20.1
14.4
P1
P3
P4
P5
P6
D2
GSG
Molecular Weight X 10^3
94
67
43
30
20.1
14.4
P1
P3
P4
P5
P6
D2
GSG
IMMUNE
P1
P3
P4
P5
P6
D2
RESISTANT

molecules between 90 to 100 kD. The indices of similarity between these materials were also low (P5/P6 = 23%, P5/D2 = 24%, P6/D2 = 49%).

Western blotting was used to identify immunogenic proteins on the pooled peaks, as well as on a female salivary gland homogenate. Sera from immune cattle were used (3.6.3) and a pool of sera of five animals of high resistance to the tick were also tested (3.1.3). Results are presented in figure 4.15.

Only fraction D2 from the larval homogenates contained a single antigen recognized by sera of the immune animals. This band showed identity with an antigen, molecular weight 39 kD recognized in GSG. The sera from the resistant animals recognized the same bands, but with a lower intensity.

4.4.- DISCUSSION

A characterization of the proteins contained in each extract and a study of the degree of separation obtained during each step of these fractionations was performed by the comparison of the electrophoretic pattern of the different mixtures of proteins (as detected by protein specific stains or by the recognition for specific antibodies) on SDS-PAGE. This discussion is primarily based on those findings. Salivary gland homogenates from either R. appendiculatus or B. microplus females were also electrophoresed for comparative purposes. No attempt was made to describe the protein composition of GSG. These homogenates were included in the gels to compare the presence of similar antigens (detected by Western blotting), between them and the larval extracts.

Salt fractionation and anion-exchange chromatography proved to be useful methods for the practical separation of tick-derived proteins. When larval extracts from either R. appendiculatus or B. microplus, or salivary glands extracts from R. appendiculatus were subjected to salt fractionation, a similar pattern of proteins was seen on materials precipitated at low ammonium sulphate concentrations (35% saturation), and on materials obtained after further concentration (55 - 65% saturation ammonium sulphate) of the supernatant obtained after the initial fractionation at 35% saturation. Higher protein yields were obtained on the precipitates at high salt concentration. Chromatographies of the later precipitates showed better repeatibility than those performed on the precipitates at low salt concentrations.

A clear separation of proteins was obtained after anion-exchange chromatography of any material on DE-52 (from both tick species or from larval or salivary gland origin). Typically few proteins of low molecular weight (below 30 kD) were eluted on the lower molarities of sodium chloride used on the solvent. In all the chromatographic separations, when higher salt concentrations were used on the solvent, proteins of higher molecular weight were liberated from the anion-exchanger. Coincidentally at molarities of sodium chloride between 150-200 mM, all the materials yielded proteins arranged in two groups (one around 43 kD and other around 70 kD).

Separation of B. microplus larval extracts using DEAE-Sephadex A-50 showed some differences with the pattern seen on DE-52. Faint bands of low molecular weight proteins were seen after electrophoresis of materials eluted at molarities of sodium chloride

up to 200 mM, but they were more abundant than those separated on DE-52. Molarities of sodium chloride between 300-500 mM were required for the elution of the proteins grouped around 43 and 70 kD when the B. microplus larval extracts were fractionated on DEAE-Sephadex A-50. Note that proteins grouped similary were eluted from DE-52 at only 150-200 mM of sodium chloride in the eluant buffer.

A direct comparison of the results obtained during these separations with those reported using similar methods, is difficult because each reported experiment had different final objectives, used different methods and used various approaches to identify the relevant molecules. So, whilst Binta and Cunningham (1984) used step changes of molarity of sodium chloride to separate R. appendiculatus larval extracts on DE-52 and split their material into two peaks of protein according to the protein concentration of the eluant, Willadsen and Williams (1976) used a linear gradient of sodium chloride on DEAE-cellulosae to separate B. microplus larval extracts and segregated their materials according to the esterase activity and to the antigenicity (measured as the size of the reaction produced by the injection on cattle exposed to the tick) of each fraction produced after chromatography.

This experiment used a combination of those methods. Fractions eluted after chromatography were pooled into major peaks according to the shape of the curve of protein concentration and to the molarity of sodium chloride at which they were eluted. The allergenic activity of each of the separated materials was studied by their intradermal injection into animals exposed to the respective tick. The type of proteins contained on each peak were

also studied by the electrophoretic separation and staining for protein, and by the subsequent recognition of these proteins by sera from immune animals.

In the R. appendiculatus-rabbit laboratory model, many proteins derived from total larval extracts were recognized by sera from immune animals. Of these, five showed identity with proteins contained in salivary gland homogenates from the same tick and three bands of protein not detected on the salivary gland homogenate were present in materials eluted at 150 and 200 mM of sodium chloride. These proteins could be the same proteins isolated by Binta, Mugera and Mushi (1985) who indicated good immunogenic activity on rabbits of a larval material separated by anion-exchange chromatography at high molarities of sodium chloride.

On the other hand on the B. microplus and cattle, few proteins either from larval or salivary gland origin were recognized by sera from immune cattle. A band of molecular weight about 39 kD was constantly recognized in the complete larval extracts and very faintly in materials eluted at high molarities of sodium chloride during chromatography on DEAE-Sephadex. This band showed identity with a protein recognized on the salivary gland homogenate.

The isolation from larval extracts of an esterase of molecular weight of 60 kD at 120 mM sodium chloride and of a proteolytic enzyme inhibitor of molecular weight of 18.5 kD at 50 mM sodium chloride both displaying allergenic activity have been described (Willadsen and Williams, 1976; Willadsen and Riding, 1979). Whether these proteins were contained on the materials separated during these experiments, is not known.

It must be remembered here, that the final objective of this

experiment was not the ultimate purification of a single protein for biochemical purposes, but the practical separation (on forms and quantities suitable for field use) of tick-derived proteins that showed the capacity to elicite hypersensitive reactions on animals exposed to the tick. It was expected that the degree of reactivity to those materials on individual animals could be related to their capacity to display resistance to the parasite.

The results of the estimation of the capacity of each semi-purified material to induce hypersensitivity reactions on the animals are described in the following chapters. The relevance of those findings to the final objectives of this study will be studied in the general discussion.

CHAPTER FIVE:

PRELIMINARY EVALUATION OF SKIN TEST METHODS USING A SMALL ANIMAL MODEL AND A THREE-HOST TICK.

SUMMARY

Acquisition of resistance to the brown ear tick Rhipicephalus appendiculatus was elicited in different groups of rabbits by three sequential artificial infestations of each of the various instars of the tick or to all of them. The level of resistance acquired by these rabbits and by a group of control rabbits not previously exposed to the tick, was tested a month later by a challenge infestation with 100 nymphs and 8 adult females. Two days before the beginning of the final infestation the animals were skin tested using as antigens proteins isolated from larvae or contained in salivary gland suspensions.

Rabbits acquired resistance to the tick after any instar had fed on them. The resistance was expressed as reduction of engorged weights, reduction of the proportion of ticks reaching maturity, and reduction of the egg laying capacity of the females. The control group showed significantly higher values than the other groups of all the parameters. Only minor differences were seen between the tick exposed groups, showing a tendency to produce smaller values of the parameters of tick feeding success, when the immunization exposure of the group was homologous with that on which the challenge parameter was calculated.

Skin test responses varied according to the type of protein used. Those elicited by the salivary gland homogenate were stronger

at 24 hours post injection and showed reduction at 48 hours. These reactions were characterized by cellular infiltrates consisting mainly of neutrophils. The reactions elicited by the proteins extracted from larvae and separated by anion-exchange chromatography displayed wide variation between animals. Two of the isolates elicited reactions characteristic of delayed hypersensitivity. The level of the reactions on the control group could not be separated from those of the animals exposed to ticks, demonstrating that the isolates contained proteins that had the ability to be mediators of inflammation. The relationship between the level of resistance of the rabbits to the tick and the size of the reaction elicited by different allergens were assessed by single correlation analysis and by multiple regression analysis. It was shown that the level of reactivity to the salivary gland homogenate at 24 hours post inoculation, is related to the capacity of the animals to prevent the successful feeding of the tick. On the basis of these results it can be said that GSG is still the best source of proteins for the skin test purposes.

5.1.- INTRODUCTION.

Acquired resistance to ticks has an immunological basis consisting of cell mediated, antibody mediated and complement dependent effector mechanisms (Willadsen, 1980a), but the type of immune response involved seems to change between different hosts and between different species of ticks used for the studies (Wikel and Allen, 1982).

Laboratories that study the problem of immunity to ticks, have

used different laboratory models, using mainly guinea pigs and rabbits, but also sometimes cattle as hosts, and different tick species including Rhipicephalus appendiculatus, Amblyomma americanum, Dermacentor andersoni and Boophilus microplus (Allen and Humphreys, 1979; Brown, Barker and Askenase, 1984; Kemp et al., 1976; Schleger et al., 1976; Shapiro, Voigt and Fujisaki, 1986). However, few of these laboratories have examined the problem of cross resistance to different species of ticks, or the effect of resistance to different instars of the same species.

There are no small animal laboratory models available for studies on B. microplus, which feeds mainly on cattle (Willadsen, 1980a). Cattle are difficult and expensive to maintain as a laboratory animal, and no laboratory colony of B. microplus is maintained at the CTVM.

When resistance to a one host tick as B. microplus is studied on its natural host, usually for practical purposes there is no necessity to make distinctions between resistance developed to different instars of the same tick. This matter becomes important when the study of a three host tick is faced, or when they are used as models. In this context, cross resistance between instars of B. microplus is equivalent to cross resistance between instars of R. appendiculatus (Walker, Fletcher and Todd, 1989).

The purpose of this preliminary experimentation was to develop methods for the isolation of antigens from larvae of ticks and methods for the development of skin tests for selection on the basis of resistance to ticks, that could be adapted to the conditions prevailing and to the species of ticks in Colombia, for use on large

numbers of cattle. The laboratory colony of the tick R. appendiculatus that was available at the CTVM, was selected as a laboratory model for preliminary experimentation. In other words, during this study it was intended to confirm the hypothesis that the existing methods of antigen preparation would give suitable reactions, and to establish suitable means of using the antigens and measuring and analysing the data of skin test reactions in relation to resistance to ticks.

5.2.- MATERIALS AND METHODS.

5.2.1.- Rabbits.

Five groups of New Zealand white female rabbits were used. Each group of three rabbits was subjected to repeated infestations with the appropriate instar of the tick as shown in table 5.1. This was the immunization scheme. Due to the difficulties involved in the handling and infestation with ticks of large groups of animals the exposure of the rabbits to the ticks was arranged in sequence as shown in table 5.2.

The challenge infestation consisted of a single infestation with 100 nymphs on the left ear and 8 females plus four males on the right ear. Males were introduced to ensure fertilization of females and thus engorgement. They were kept to a minimum in order to reduce the damage to the ears of the rabbits.

For each group the skin test was performed three weeks after the last infestation for immunization, and the challenge infestation was initiated 48 hours after the intradermal injections. Control rabbits were only brought to the animal room a few days before the

Table 5.1. Regime of exposure to ticks in order to immunize groups of rabbits against different instars of the tick R. appendiculatus and regime of the challenge infestation.

GROUPS	Number of Rabbits	IMMUNIZATION SCHEDULE*	CHALLENGE**
1	3	Larvae, Nymphs, Adults. Then the three stages at once	8 Females + 4males 100 Nymphs
2	3	Three infestations with approx. 1000 larvae	8 Females + 4males 100 Nymphs
3	3	Three infestations with 100 Nymphs	8 Females + 4males 100 Nymphs
4	3	Three infestations with 8 females + 4 males	8 Females + 4males 100 Nymphs
5	5	Controls. No exposure to ticks.	8 Females + 4males 100 Nymphs

* Each single infestation was performed a week after the end of the previous infestation

** One month after the last immunization infestation a skin test was performed using proteins derived from larvae or a homogenate of salivary glands from adults and the following day the ticks of the challenge infestation were applied.

Table 5.2. Chronology of tick infestation and skin test activities related to the experiment "Preliminary evaluation of skin test methods using a small animal model and a three host tick".

	Weeks																	
RABBIT GROUPS	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
GR-1 :							L		N	A			LNA					CH
GR-2 :	L		L			L					CH							
GR-3 :		N			N		N					CH						
GR-4 :			A			A				A					CH			

Symbols for the ticks applied to the rabbits

L = approx. 1000 larvae
N = 100 nymphs
A = 8 females + 4 males
LNA = The three instars at a time

CH = Challenge infestation with 100 nymphs and 8 females + 4 males. Skin test.

skin test and the final challenge infestation. One control rabbit was tested with each experimental group except for group three where two controls were included.

Animals were kept and managed according to the sanctions of the Cruelty to Animals Act, 1876 of the United Kingdom.

5.2.2.- Ticks.

The laboratory colony of the tick R. appendiculatus maintained at the CTVM provided the larvae, nymphs and adults ticks required for the experiment. The ticks originated from Kenya, and have been maintained by feeding on rabbits and cattle.

All experimental ticks were derived from ticks fed on rabbits. Oviposition and hatching or moulting were allowed at 25° C and then the ticks were stored at 18° C, and 85% relative humidity until used. All ticks were used within no more than a four months period of storage.

Due to the experimental protocol (table 5.2), not all the ticks used belonged to the same batches, but all the previous feeds were made on rabbits not exposed to ticks. Ticks were applied to the ears inside protective bags of cotton as described by Bailey (1960) and Irvin and Brocklesby (1970).

5.2.3.- Determination of tick feeding success.

During the challenge infestation the ears of the rabbits were checked every day, and for each individual rabbit and for each instar the following data were recorded:

- Number of ticks not attached at 24 hours.
- Number of ticks detached naturally per day.
- Number of engorged ticks detached by the limit day.
- Average weight at engorgement.
- Total nymphal weight.
- Total adult weight.

The limit for engorgement of nymphs was set on the 7th day post infestation. For adults the limit was extended to 10th day.

The weight of the ticks was determined as follows:

For nymphs, total numbers detaching per rabbit per day were washed, dried over plaster, weighed and then counted. The data of weight from all days were added (Total nymphal weight) and divided by total numbers to obtain an average engorgement weight per rabbit.

For adults, the data of engorged females were recorded only. These were washed, dried over plaster and weighed individually. The sum of the weights of all the females fed on a single rabbit constituted the total adult weight.

All females fed on the same rabbit were grouped and allowed to oviposit at 25 °C for three weeks. Then the eggs produced were weighed, and data recorded as total egg mass. This was divided by the number of ticks ovipositing to produce the average weight of the egg masses produced by the female.

In order to simplify the calculations, the comparisons of tick feeding success between different individuals and different groups, and the analysis for relationships with the skin test results were only performed with three parameters that summarize all the aspects

on which resistance could influence the tick feeding. These parameters were: total nymphal weight, total adult weight and total egg mass.

Resistance to ticks was calculated individually using the following formula:

$$R = 1 - \left[\frac{Nc}{Nn} \times \frac{Ac}{An} \times \frac{Ec}{En} \right]$$ where:

R = resistance (x 100, if expressed as percentage)

Nc = total nymphal weight at challenge infestation

Nn = total nymphal weight in naive animals

Ac = total adult weight at challenge infestation

An = total adult weight in naive animals

Ec = total egg mass at challenge infestation

En = total egg mass in naive animals

As no naive values for each animal were available, data from all animals were compared with a mean of the control group.

5.2.4.- Skin test.

5.2.4.1.- Proteins used as inocula.

Three weeks after the last application of ticks to the rabbits, an intradermal test was performed using as inocula corresponding concentrations of the proteins listed below. All materials were prepared beforehand and freeze dried. On the day of the test, corresponding aliquots were diluted in distilled water at a concentration of 100 µg/ml of protein in water. All tests were

performed using a fixed volume of 100 µl per inoculation site. Protein concentration was measured using the Bicinchoninic acid method available as a commercial kit (BCA Protein Assay Reagent, Pierce & Warriner Ltd.,UK).

The following were the proteins used for the skin test:

- GSG.- A homogenate of salivary glands prepared as described in chapter three (3.3), adjusted on protein concentration and freeze dried.
- PK1.- Larval proteins separated by anion exchange chromatography (4.3.1) and corresponding to those eluted at a 50 mM sodium chloride in the eluant buffer.
- PK2.- Larval proteins eluted between 75-100 mM sodium chloride in the eluant during chromatographic fractionation.
- PK3.- Proteins separated at 150 mM sodium chloride.
- PK4.- Proteins extracted from larvae at 200 mM sodium chloride in the eluant.
- PBS.- Used as control.

5.2.4.2.- Protocol used for the skin test.

On each day in which the skin test was performed, the following procedure was conducted on all the rabbits including the controls.

- The previous day the skin was shaved.
- The skin was disinfected with 70% alcohol, and squares (4x4 cms) were marked out with an indelible pen.
- The test site was assigned at random.
- The pre- test skin thickness was measured at each testing site, quoting it as the reading of a skin fold with the calliper.
- Proteins and controls were injected intradermally at hour 0.

- 30 minutes post-injection the area of wheal reaction was measured in two diameters (anterolateral and dorsoventral), and their product recorded as area of reaction.
- 4 hours post injection (HPI) the skin thickness and the area of reaction were measured, making the reading in the places where the wheal and erythema were evident.
- 24 HPI, reactions of induration and erythema were measured and quoted as skin thickness and area of reaction.
- 48 HPI the same reactions were recorded.

Data of reactions to each allergen on individual rabbits were recorded as area of reaction and as increase in skin thickness calculated for each individual injection site as the result of the skin thickness obtained minus the skin thickness before the injection was applied (0 hours).

5.2.5.- Skin biopsies

A rabbit previously exposed to all instars of R. appendiculatus ticks was used to study the morphological changes associated with the reactions to the antigens. Responses to GSG, PK1, PK2, and PBS as control were observed. For each antigen three inoculations were performed, following the same methods described for the skin test.

Skin biopsies were taken in the centre of the reaction at 30 minutes post injection in one site, at four hours post injection in another and at 24 hours in the remaining site. Procedures for taking and processing the biopsies, and methods followed for the cell counts are described in chapter three (3.7).

5.2.6.- Examination of sera for antibodies to salivary antigens by ELISA

Blood from all the experimental rabbits was collected two weeks after the skin test, when the challenge infestation was finished and sera were obtained and kept frozen. In 1989, an ELISA test was conducted on the sera (3.6), to compare the antibody reactivity to GSG of the rabbits and relate that with the skin test results and with the displayed resistance.

5.3.- RESULTS.

5.3.1.- Tick feeding performance.

The different records of feeding performance of the ticks on the individual rabbits of various groups are shown in tables 5.3 and 5.4. In most of the parameters, strong differences between the previously tick exposed and the control groups were noted, mainly in the average weight at engorgement for nymphs and adult females and the feeding duration of nymphs. A strong effect of resistance was also noted on the egg laying capability of the females as determined by the average egg mass per female. However, resistance to ticks does not seem to affect all the individual ticks to the same extent. Figure 5.1 illustrates this fact. All ticks feeding on naive rabbits displayed a similar range of engorgement weights and showed similar colours, whilst ticks feeding on resistant rabbits displayed increased variance of data and showed a different pattern of colours from normal (table 5.4).

Table 5.5 displays the compiled results for all rabbits of the three parameters selected for tick feeding success, compared with the calculated resistance. It is interesting to note the individual

Table 5.3. Records of various events of the feeding performance of nymphs of the tick R. appendiculatus, fed on rabbits previously exposed to repeated infestations with different instars and on controls naive to ticks. The challenge consisted of an infestation with 100 nymphs and 8 adult females applied one month after the final exposure to ticks.

		NYMPHS						
GROUP	RABBIT	% not attached at 24 hours	% engorged by day 7th	Average weight at engorgement (mgs)	Duration of engorgement No of ticks detached by day 4	5	6	7
1 MIXED	1A	11	73	4.68	6	28	27	12
	1B	26	40	3.24	2	16	13	9
	1C	12	74	3.96	5	24	28	17
2 LARVAE	2A	3	61	4.57	-	16	23	32
	2B	17	61	5.26	-	9	29	23
	2C	9	52	4.01	2	3	13	34
3 NYMPHS	3A	12	77	3.36	20	27	14	16
	3B	18	30	3.31	-	6	10	14
	3C	37	42	3.56	3	14	11	14
4 ADULTS	4A	20	76	4.89	3	34	23	16
	4B	10	72	4.45	3	35	29	5
	4C	30	42	3.75	-	6	28	8
5 CONTROL	Co1	16	84	8.70	9	38	32	5
	Co2	5	93	9.17	-	33	43	17
	Co3	3	93	9.25	4	67	22	-
	Co4	16	83	7.58	3	58	19	3
	Co5	6	92	9.65	8	75	9	-

Table 5.4. Records of various events of the feeding performance of adult females of the tick R. appendiculatus, fed on rabbits previously exposed to repeated infestations with different instars and on controls naive to ticks. The challenge consisted of a infestation with 100 nymphs and 8 adult females applied one month after the final exposure to ticks.

		ADULTS (females)								
GROUP	RABBIT	No. not attached at 24 hours	No. engorged by day 10th	Average weight at engorgement.(Range) (mgs)	Average egg mass by female (mgs)	Duration of engorgement No of ticks detached by day 6	7	8	9	10
1 MIXED	1A	0	7	131.1 (32 - 360)	56.7	-	2	2	1	2
	1B	3	4	158.4 (24 - 277)	102.9	-	1	1	1	1
	1C	4	4	46.3 (31 - 76)	14.5	-	-	-	2	2
2 LARVAE	2A	0	3	158.3 (51 - 306)	58.0	-	-	-	2	2
	2B	2	3	179.5 (28 - 301)	77.2	-	-	-	1	2
	2C	2	2	80.3 (33 - 128)	35.3	-	-	-	-	2
3 NYMPHS	3A	0	7	109.1 (28 - 172)	41.6	-	3	1	2	1
	3B	4	2	73.3 (50 - 96)	20.7	-	-	1	1	-
	3C	6	1	131.1	63.0	-	-	-	1	-
4 ADULTS	4A	3	4	50.2 (26 - 87)	13.2	-	-	-	-	4
	4B	1	6	49.3 (12 - 120)	28.2	-	-	1	2	3
	4C	3	4	25.3 (7 - 58)	8.1	-	-	-	1	3
5 CONTROL	Co1	2	6	421.8 (350 - 483)	175.3	-	1	1	2	2
	Co2	0	5	296.3 (200 - 344)	148.7	-	-	-	5	-
	Co3	2	6	369.3 (309 - 407)	170.5	1	-	2	2	1
	Co4	4	2	268.8 (110 - 427)	134.9	-	-	-	1	1
	Co5	1	6	399.6 (329 - 463)	171.5	-	1	3	2	-

Figure 5.1. Comparison of the shape and colour of naturally detached Rhipicephalus appendiculatus adult female ticks fed on naive (Control 1) or resistant (1A and 1C) rabbits.


CONTROL 1


Rabbit 1A


Rabbit 1C

Table 5.5. Results of three tick feeding success parameters of nymphs and adult females R. appendiculatus ticks fed on rabbits naive (Control) or previously exposed to different instars (Groups 1,2,3 and 4) of the tick, compared to the calculated resistance of each rabbit to the tick.

GROUP	RABBIT	TOTAL NYMPHAL WEIGHT (mgs)	TOTAL ADULT WEIGHT (mgs)	TOTAL EGG MASS (mgs)	% RESISTANCE
	1A	341.9	917.7	397.0	89.96
1	1B	129.5	633.7	308.9	97.96
MIXED	1C	293.2	185.4	43.4	99.81
	2A	278.6	633.1	232.0	96.70
2	2B	321.2	538.4	231.5	96.77
LARVAE	2C	208.7	160.6	70.5	99.80
	3A	259.0	764.0	291.0	95.36
3	3B	99.4	146.5	41.5	99.95
NYMPHS	3C	149.5	131.1	63.0	99.90
	4A	371.9	200.8	39.5	99.76
4	4B	320.5	295.8	84.4	99.35
ADULTS	4C	157.7	101.4	16.1	99.98
	Co1	879.9	2530.6	1052.0	N.A.
5	Co2	853.3	1481.4	743.5	N.A.
	Co3	860.1	2215.8	1023.0	N.A.
CONTROL	Co4	628.9	537.6	269.8	N.A.
	Co5	887.6	2397.7	1029.0	N.A.

N.A.= Not applicable in this group

behaviour of tick feeding performance on rabbit control 4 (Co4), which in table 5.5 demonstrates low values in its tick feeding success performances. If the formula for resistance was applied this rabbit would show a resistance of 92%. When data was traced back (tables 5.3 and 5.4), it was found that low values in the compiled parameter were due to high numbers of ticks not attaching at 24 hours. Also adult and nymphal average weights were low but near normal. Simmilary rabbit Co2 showed a tendency to produce longer feeding periods for adult ticks. On this rabbit, only 5 of 8 attached females had detached naturally by day 10, and those detached showed lower engorgement weights, even though those weights were higher than those of resistant animals.

Such natural variation in attachment rates of ticks on rabbits is usual (Walker, A.R., personal communication, 1988), but this finding illustrates the difficulties faced when measuring resistance that is the result of a complex interaction of different mechanisms on a single compiled numerical parameter.

Differences in the responses of tick feeding success parameters according to the different groups of rabbits are displayed in figure 5.2. For each parameter data was subjected to analysis of variance, and group means were compared by a Duncan's multiple range test (Little and Hills, 1976).

Results of total adult weight and total egg mass were found to be not normally distributed and not with homogeneous variances between groups. Figure 5.3 shows how a logarithmic transformation ($\log_{10}$) proved useful for normalization of the parameter, in the graphs data are presented as rankit values (Sokal and Rohlf, 1981). Data on total nymphal weight displayed homogeneous variances between

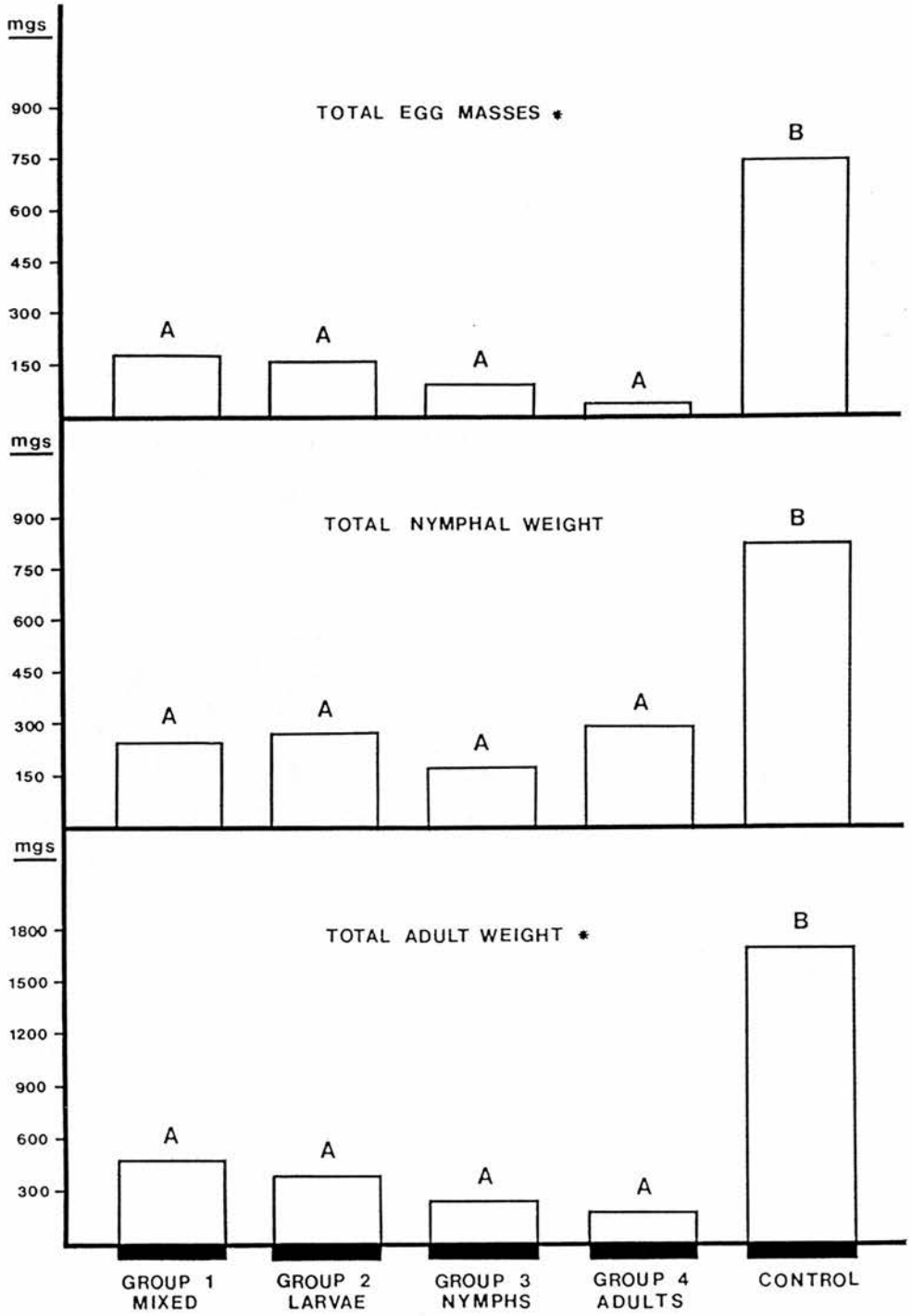


Figure 5.2. Comparison of group means of tick feeding success parameters obtained when similar numbers of nymphs and females Rhipicephalus appendiculatus ticks were allowed to feed on the ears of rabbits previously exposed to different instars of the tick. Each group consisted of three rabbits but the control group had 5. Within each parameter bars bearing different letter display significant differences between the means ($P < 0.05$).

groups and also untransformed data was shown to be linear in the rankit plot when data of naive and tick exposed animals were analysed separately. For these reasons it was not transformed for calculations. This fact could be explained by the exponential increase in weight in females when they reach the final period of engorgement (Balashov, 1972), and this influences the egg mass produced by each female.

In the three parameters used as indicative of tick feeding success, significant ($P < 0.05$) differences were found between the control group and those groups previously exposed to ticks. Responses between the groups exposed to different instars of the tick could not be statistically separated, but the lowest values were always found in the group homologous to that instar on which the tick feeding success was measured. For example, the lowest total nymphal weight was in the group previously exposed only to nymphs.

Calculated resistance displayed values ranging from 89.96% to 99.98%. Data were found to be not normal (figure 5.3) and percentages of calculated resistance were transformed to the parameter transformed susceptibility, in order to make the variance independent of the mean (Utech, Wharton and Kerr, 1978) using the following expression:

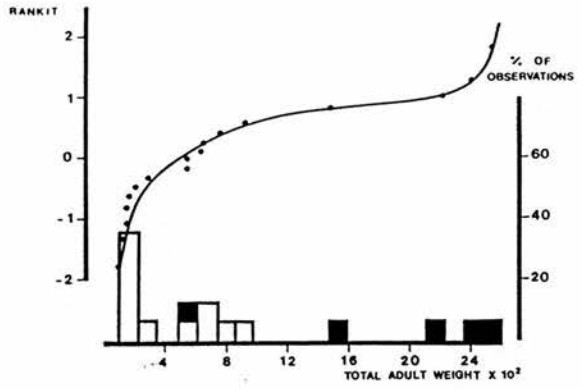
$$x' = (1 - x) \text{ EXP } 0.25 \quad \text{where:}$$

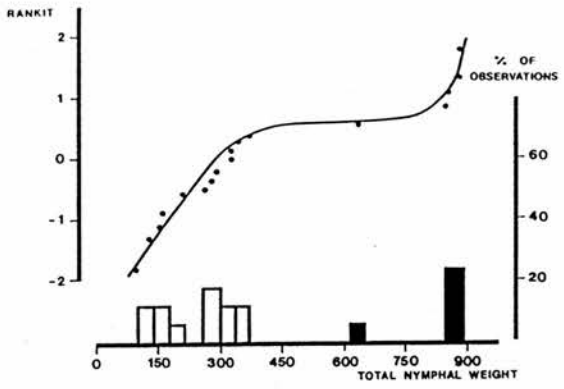
x = calculated resistance expressed as proportion of 1

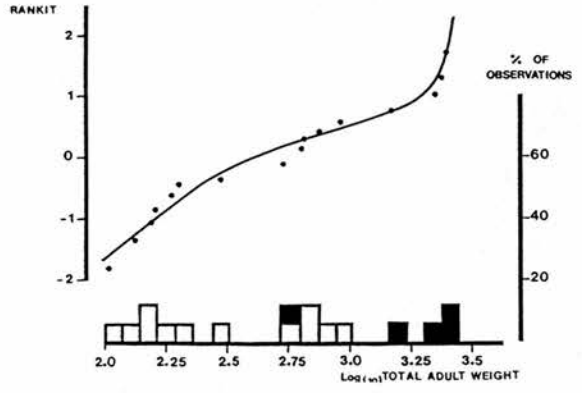
x'= transformed susceptibility value

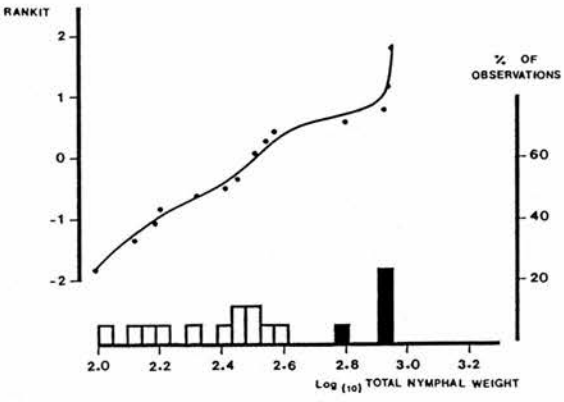
EXP = exponential power of value inside brackets

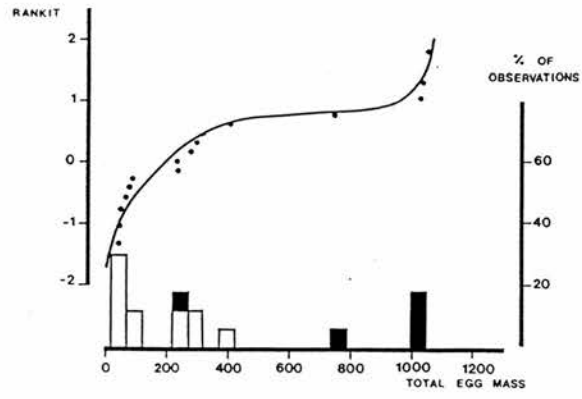
Figure 5.3. Graphic test for normal distribution of the parameters total nymphal weight, total adult weight, total egg mass and percentage of resistance to ticks, both untransformed and transformed to normalize the variable. Data distributed normally lie on a straight line when ploted as rankit values. The frequency distribution of the responses of rabbits of the control (shaded) or tick exposed groups, are illustrated in the vertical bars.

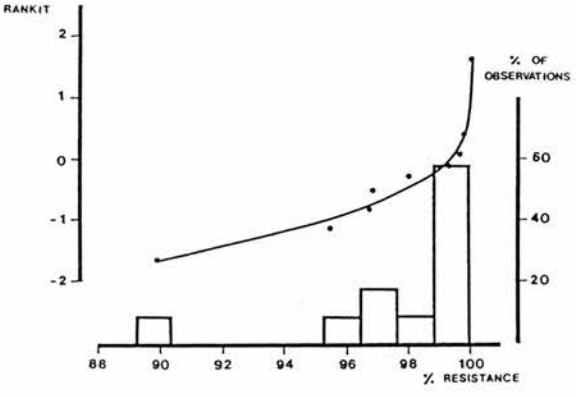

RANKIT
% OF OBSERVATIONS
TOTAL ADULT WEIGHT X 10²

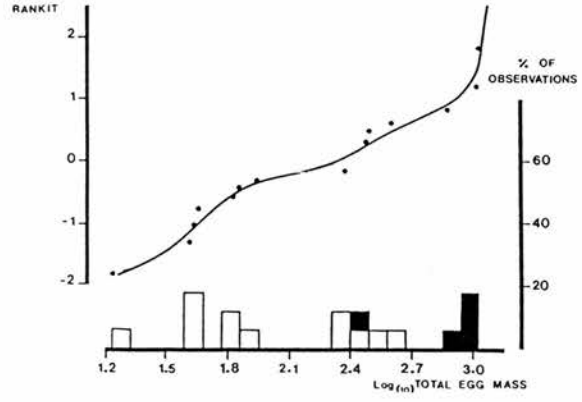

RANKIT
% OF OBSERVATIONS
TOTAL NYMPHAL WEIGHT

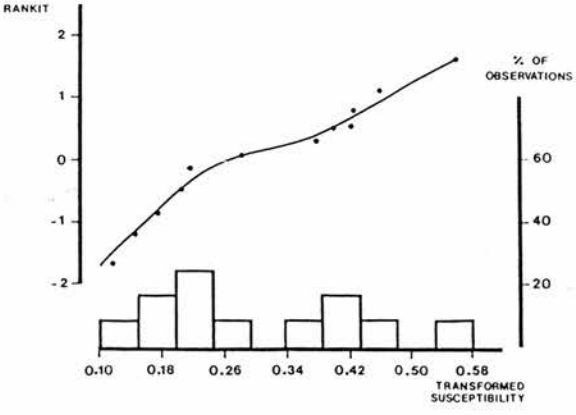

RANKIT
% OF OBSERVATIONS
Log (10) TOTAL ADULT WEIGHT


RANKIT
% OF OBSERVATIONS
Log (10) TOTAL NYMPHAL WEIGHT


RANKIT
% OF OBSERVATIONS
TOTAL EGG MASS


RANKIT
% OF OBSERVATIONS
% RESISTANCE


RANKIT
% OF OBSERVATIONS
Log (10) TOTAL EGG MASS


RANKIT
% OF OBSERVATIONS
TRANSFORMED SUSCEPTIBILITY

No significant differences between the groups exposed to ticks were found when the data of transformed susceptibility were subjected to analysis of variance.

5.3.2.- Skin hypersensitivity reactions.

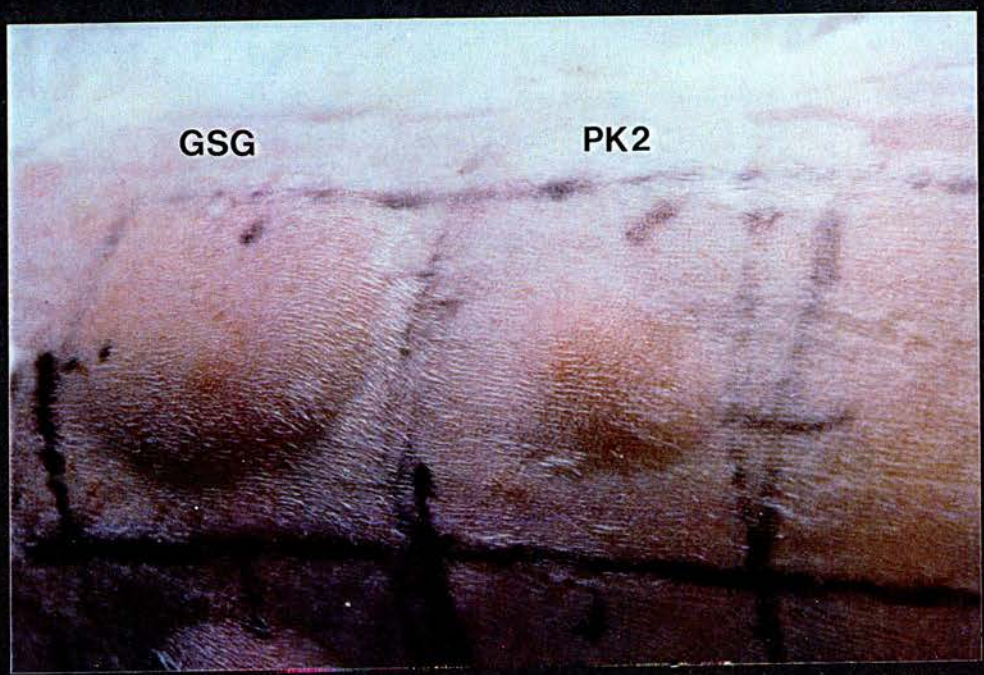
5.3.2.1.- Pattern of the evolution in time and comparison of the parameters used to measure the reactions.

Reactions produced by the different proteins when injected into the skin of rabbits, were easily identifiable and were characterized by an induration which was usually painful. The pattern of the reactions were different between the larval derived proteins and GSG. Figure 5.4 illustrates these differences, larval proteins tended to produce narrower reactions but with more prominent induration.

The evolution in time of the reactions elicited in the skin of the rabbits (naive and immune to ticks), are displayed in figure 5.5, where a comparison on the type of measurement of the reaction is performed. Note how the higher values were obtained on GSG at 24 HPI, when the area of reaction was measured, but the higher increase in skin thickness was produced by PK2 at 48 HPI.

Only slight immediate hypersensitivity reactions (Roitt, Brostoff and Male, 1985) were observed. GSG produced major reactions at 24 HPI, with a decrease in reactions afterwards. PK1 and PK2 showed slower development of reactions than GSG displaying peak values at 48 HPI. The proteins contained in chromatographic materials PK3 and PK4 elicited minor reactions that were measurable but in some parameters they were not significantly different from

Figure 5.4. Hypersensitivity responses to proteins derived from Rhipicephalus appendiculatus ticks in the skin of a resistant rabbit. Reactions at 4 hours post injection are displayed to a salivary gland homogenate in the left and to the larval PK2 extracted by chromatography in the right.


GSG
PK2

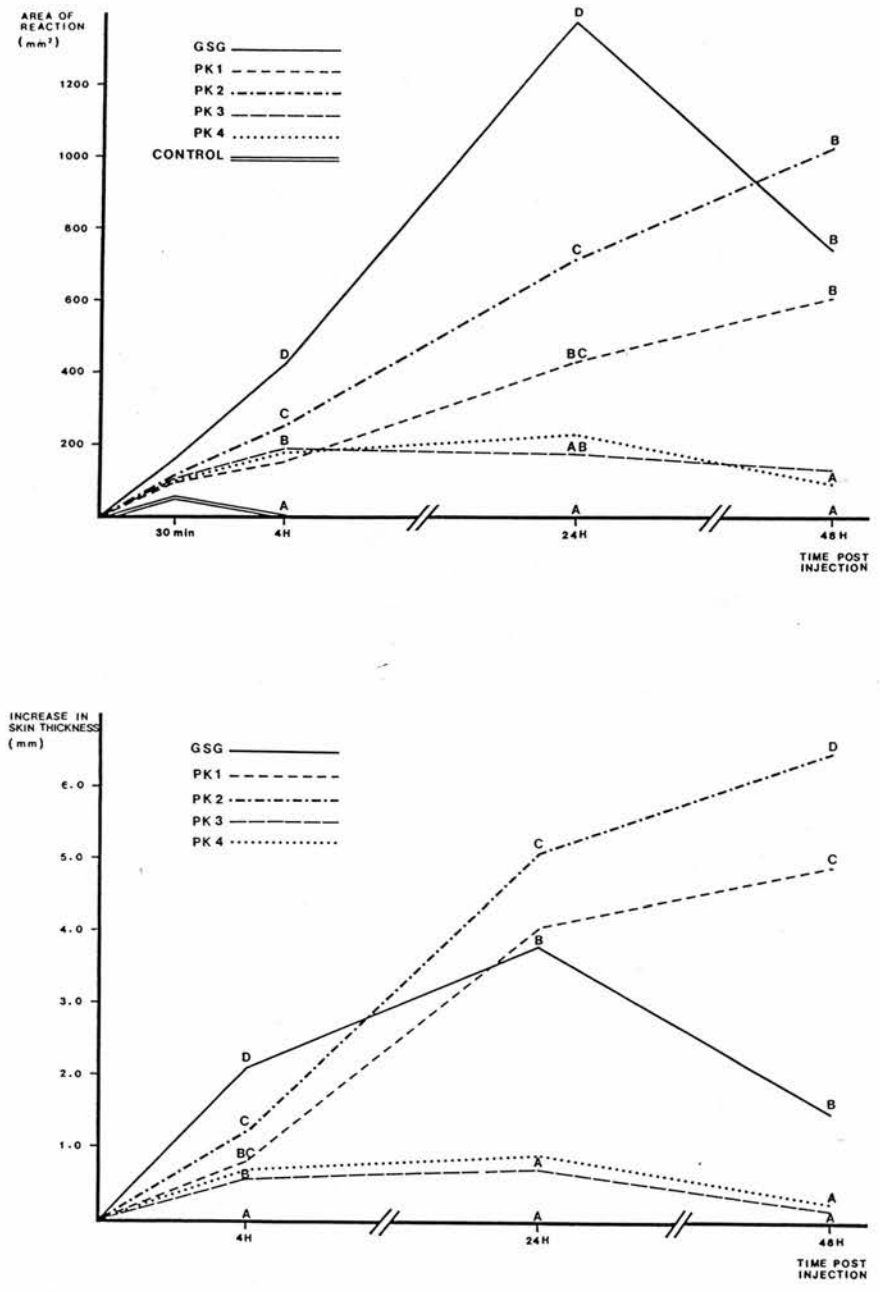


Figure 5.5. Dynamics of the hypersensitivity reactions elicited by different proteins derived from larvae or by a salivary gland homogenate from adult Rhipicephalus appendiculatus ticks in the skin of rabbits with different types of previous exposure to the tick. The top graph illustrates the means of 17 rabbits in the parameter area of reaction. The bottom graph displays the means of the increase in skin thickness of the reactions. In each parameter and each time of reading, means bearing different letters are statistically different ($P < 0.05$). Saline control readings when not specified are quoted as zero.

PBS reactions (figure 5.5).

A compiled parameter of the reaction was calculated by multiplying the area of reaction by the increase in skin thickness obtained. To avoid zero multiples, the value of 1.0 was added to all data for area of reaction and the value of 0.01 was added to all data for increase in skin thickness. Figure 5.6 shows the trend of the reactions to the different proteinaceous materials. Reactions to the salivary gland extract were significantly stronger up to 24 HPI. Reactions to the materials PK1 and PK2 got their peak level at 48 HPI.

5.3.2.2.- Comparison of the reactivity to each of the materials according to the type of previous exposure to ticks of the rabbits.

For GSG, the size and type of reactions changed according to the previous exposure to ticks of the rabbits. Figure 5.7 displays the results of the parameters: area of reaction and the increase in skin thickness, for the different groups. At 4 HPI the reactions in the tick exposed groups were significantly different from the control group, but this pattern was more marked at 24 HPI for both parameters. At 48 HPI the size of the reactions decreased but the variance increased mainly in the parameter area of reaction This was due to the subjectivity involved when measuring the area of induration, with poorly defined margins.

Data on the three parameters used to measure the reactions to GSG at 24 HPI were subjected to analysis of variance, and results are shown in figure 5.8. Differences in the responses between tick

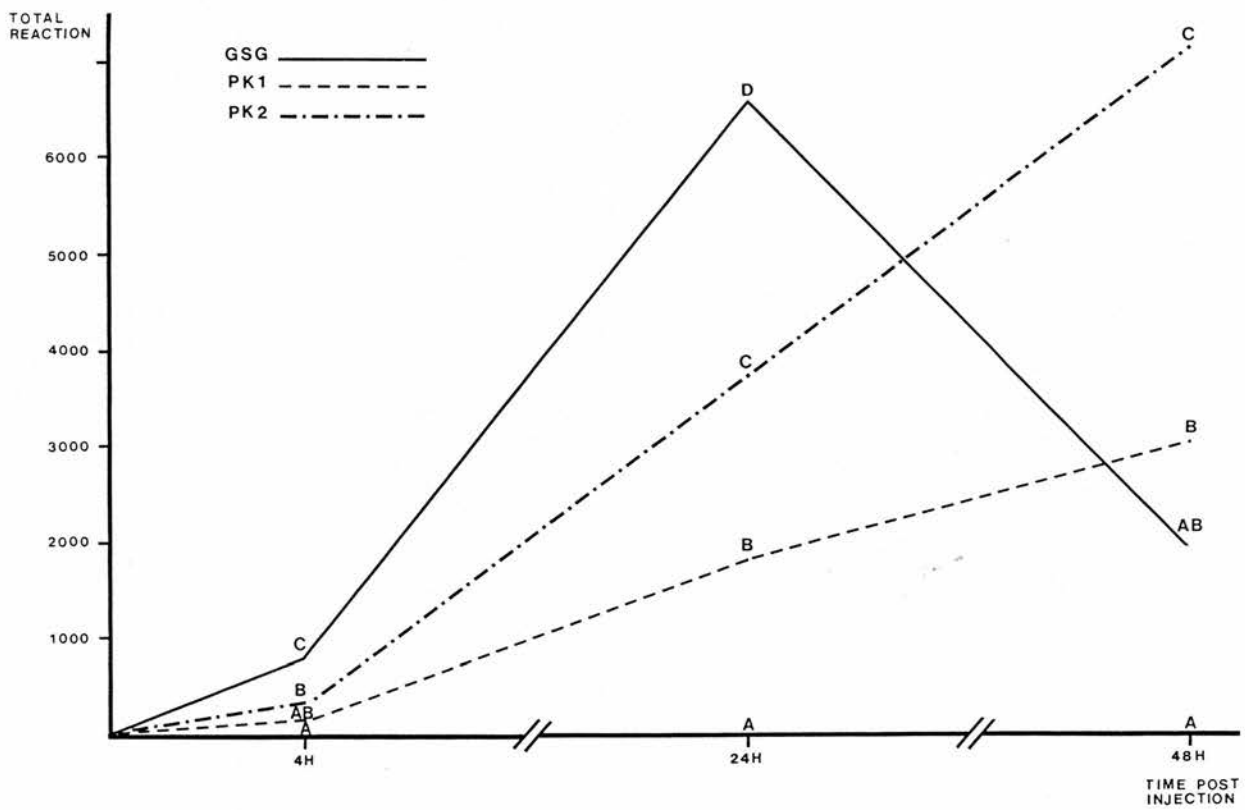


Figure 5.6. Skin hypersensitivity reactions elicited by a salivary gland homogenate from adults (GSG) and by two different mixtures of proteins extracted from Rhipicephalus appendiculatus larvae (PK1,PK2) in rabbits exposed and not exposed to different instars of the tick. Responses are quoted as total reactions. At each time of reading significant differences ($P < 0.05$) are illustrated by means bearing different letters. Responses to proteins PK3 and PK4 (not shown) were not significantly different from the saline control (A).

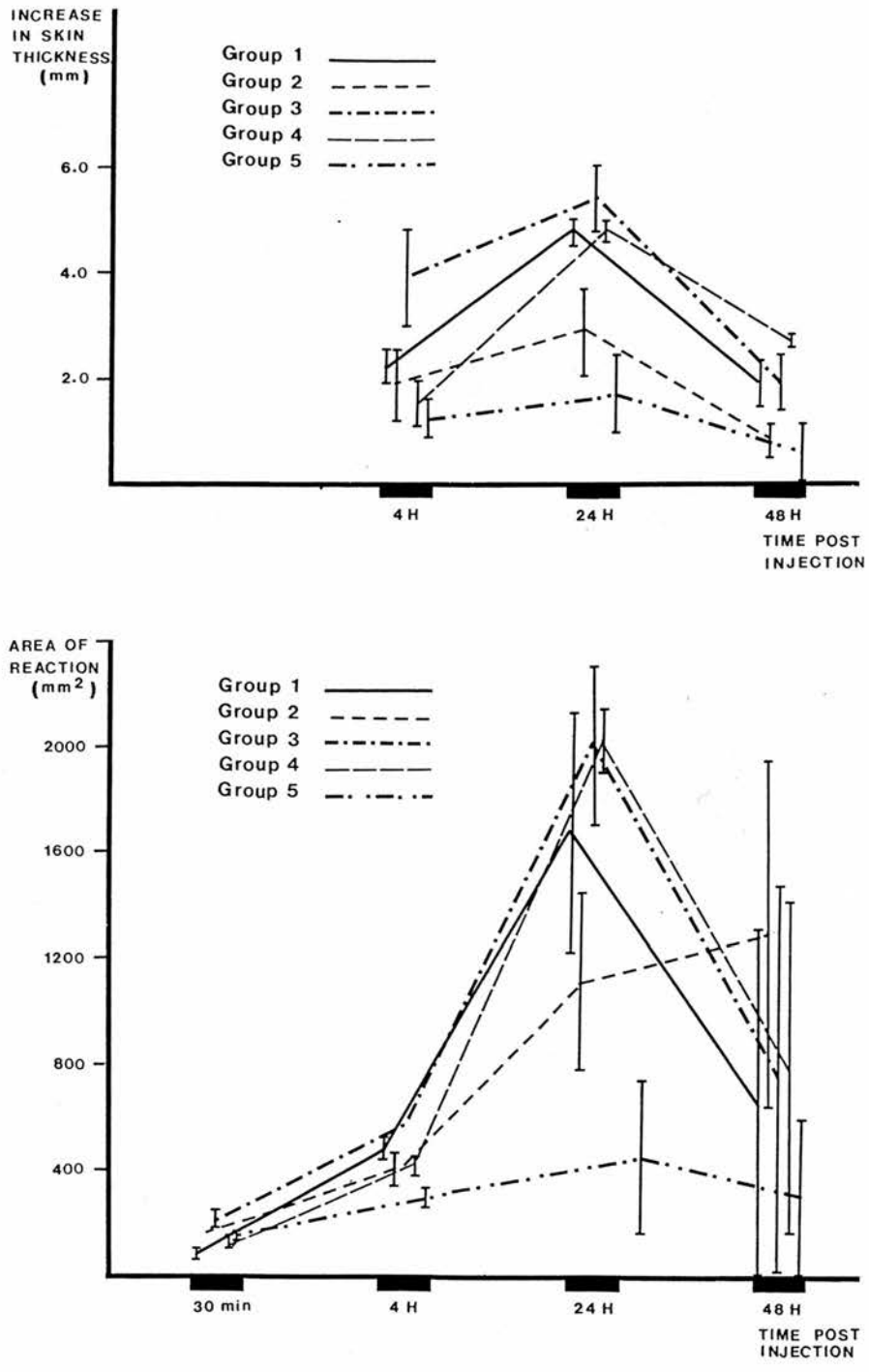
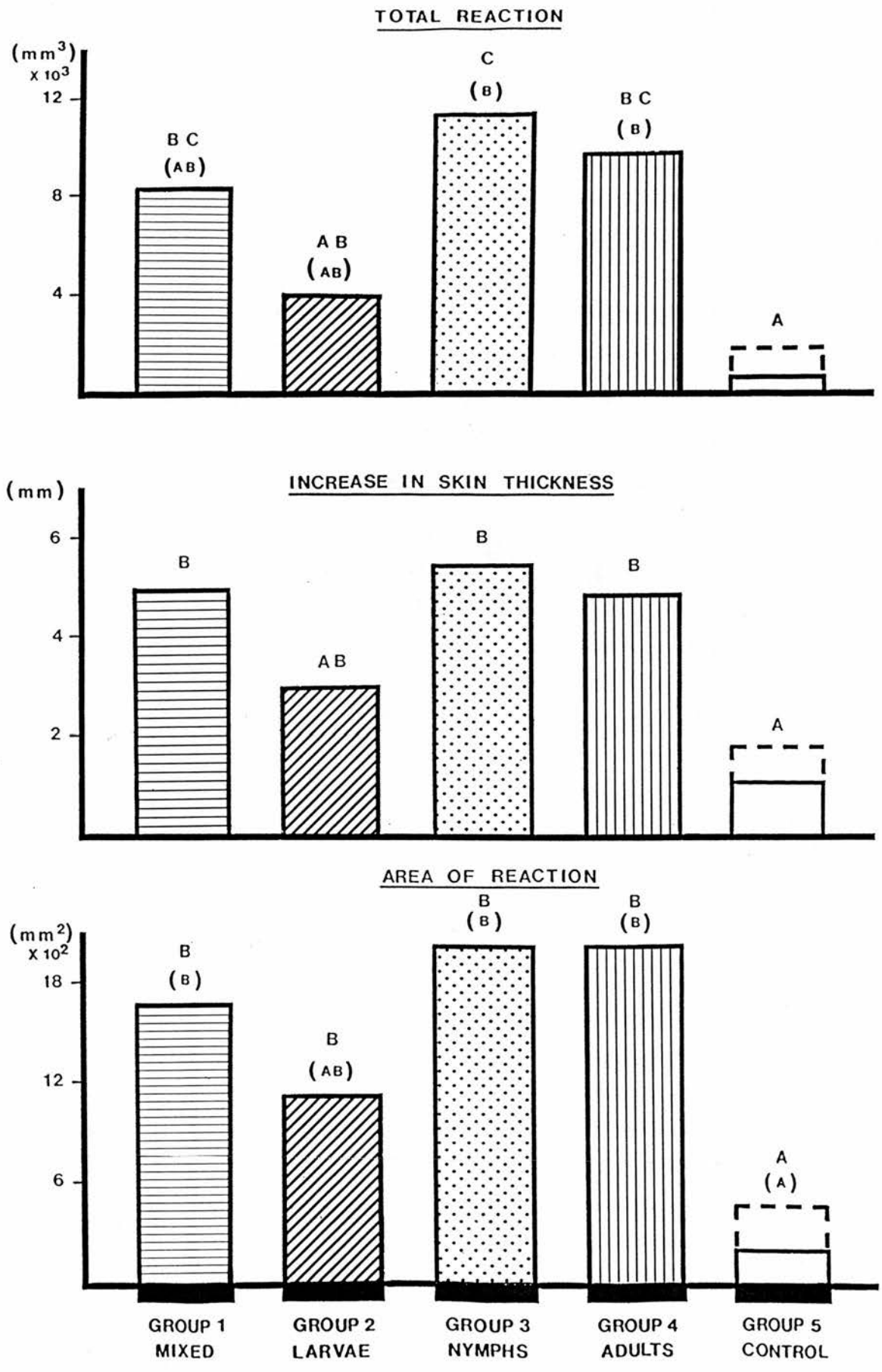


Figure 5.7. Dynamics of the skin hypersensitivity reactions elicited by a salivary gland homogenate extracted from adult female Rhipicephalus appendiculatus, when injected in rabbits with repeated previous exposure to different instars of the tick. All groups consisted of three rabbits but the control had five. The top graph illustrates the increase in skin thickness and the bottom one the area of the reactions. Vertical bars indicate standard errors. Group 1= Exposure to the three instars of the tick, Group 2= Exposure only to larvae, Group 3= Exposure to nymphs only, Group 4= Exposure to adults, Control Group= No exposure to ticks (Group 5).

Figure 5.8. Comparison of group means of three parameters used to measure reactions of skin hypersensitivity in rabbits exposed to different instars of Rhipicephalus appendiculatus ticks. Reactions displayed are those elicited 24 hours after the intradermal injection of a salivary gland homogenate extracted from female ticks. Each group was exposed three times to the indicated instar and the skin test was conducted one month thereafter. For each parameter same letters quoted on the top of the bars indicate not significant ($P < 0.05$) difference between the means. For the control group, bars drawn in dotted lines indicate the mean obtained when rabbit Co2 was included in the calculations and letters inside brackets show the corresponding changes in the multiple range test.


TOTAL REACTION
(mm³) x 10³
12
8
4
BC (AB)
AB (AB)
C (B)
BC (B)
A
INCREASE IN SKIN THICKNESS
(mm)
6
4
2
B
AB
B
B
A
AREA OF REACTION
(mm²) x 10²
18
12
6
B (B)
B (AB)
B (B)
B (B)
A (A)
GROUP 1 MIXED
GROUP 2 LARVAE
GROUP 3 NYMPHS
GROUP 4 ADULTS
GROUP 5 CONTROL

exposed and control rabbits are clearly seen in both parameters, but the links varied according to the parameter. High values in the control group were not expected and this required a closer look at the data. It was found that the rabbit identified as Co2, displayed stronger reactions than those observed in other animals of the control group. Values for area of reaction, increase in thickness and total reaction at 24 HPI to GSG for this rabbit were respectively, 1513, 4.51 and 6824; whilst the means for the other animals of the control group were, 179, 1.01 and 288. The exclusion of data from this animal reduced the variances and facilitated the grouping of the data. However, both results are illustrated.

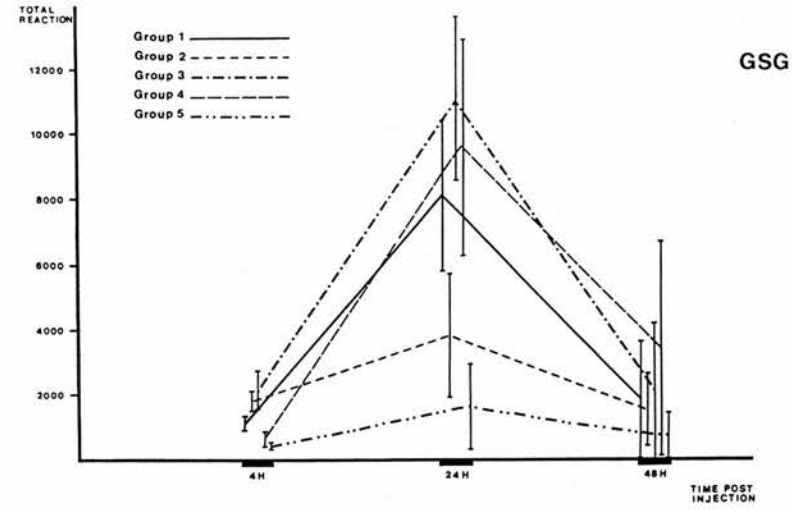
Reactions in the group of rabbits exposed only to larvae were lower than those of the other tick exposed groups but the differences were only significant in the parameter total reaction.

For the larval extracted antigens PK1 and PK2, differences in the responses between naive and tick exposed rabbits could not be demonstrated. Comparative results to GSG, PK1 and PK2 on the parameter total reaction are illustrated in figure 5.9. Note how the larval antigens produced peak reactivity at 48 HPI, with stronger responses to PK2. A slight discrimination in two types of responses can be observed for PK2 at 48 HPI. This finding will be discussed when examining the relationship with resistance.

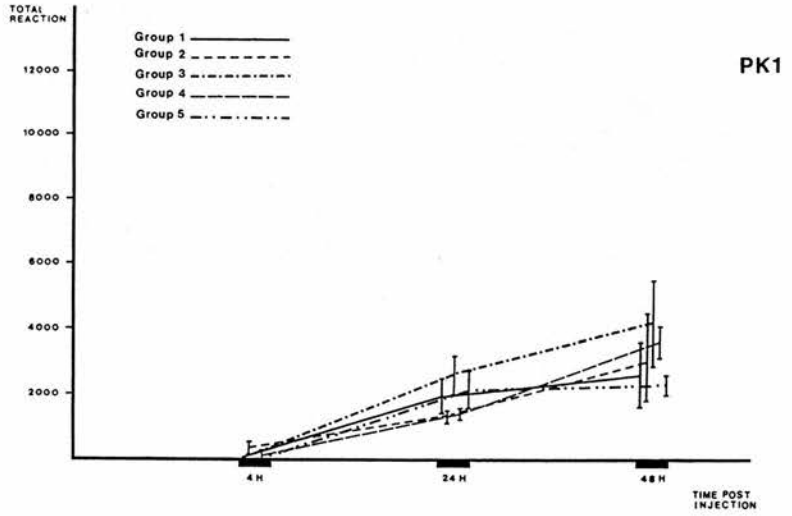
5.3.2.3.- Cellular changes associated with the hypersensitivity responses.

Following the methods described in chapter three (3.7), the differential counts for infiltrated cells in the dermis in all the

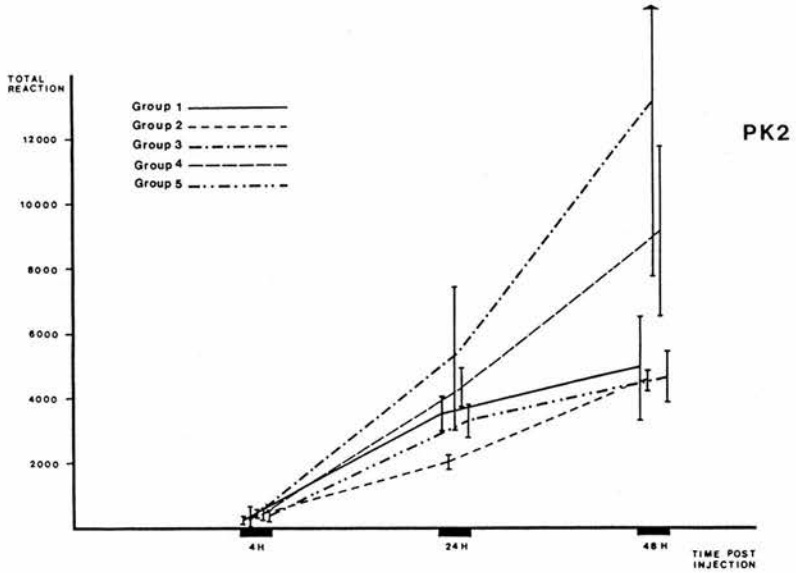
Figure 5.9. Dynamics of the skin hypersensitivity reactions to three different antigens derived from Rhipicephalus appendiculatus ticks, and measured as total reaction. The mean and standard error of reactions in 5 groups of rabbits with different types of previous exposure to the tick are displayed. The exposure to ticks of each group was as follows: Group 1, exposure to the three instars of the tick; Group 2, exposure only to larvae; Group 3, exposure only to nymphs; Group 4, exposure only to adults; Group 5, control, no exposure to ticks. The top graph illustrates responses to a salivary gland homogenate extracted from adult females (GSG). The intermediate (PK1) and bottom (PK2) graphs illustrate responses to larval proteins purified by anion-exchange chromatography.


TOTAL REACTION
Group 1
Group 2
Group 3
Group 4
Group 5
12000
10000
8000
6000
4000
2000
4H
24H
48H
TIME POST INJECTION

GSG


TOTAL REACTION
Group 1
Group 2
Group 3
Group 4
Group 5
12000
10000
8000
6000
4000
2000
4H
24H
48H
TIME POST INJECTION

PK1


TOTAL REACTION
Group 1
Group 2
Group 3
Group 4
Group 5
12000
10000
8000
6000
4000
2000
4H
24H
48H
TIME POST INJECTION

PK2

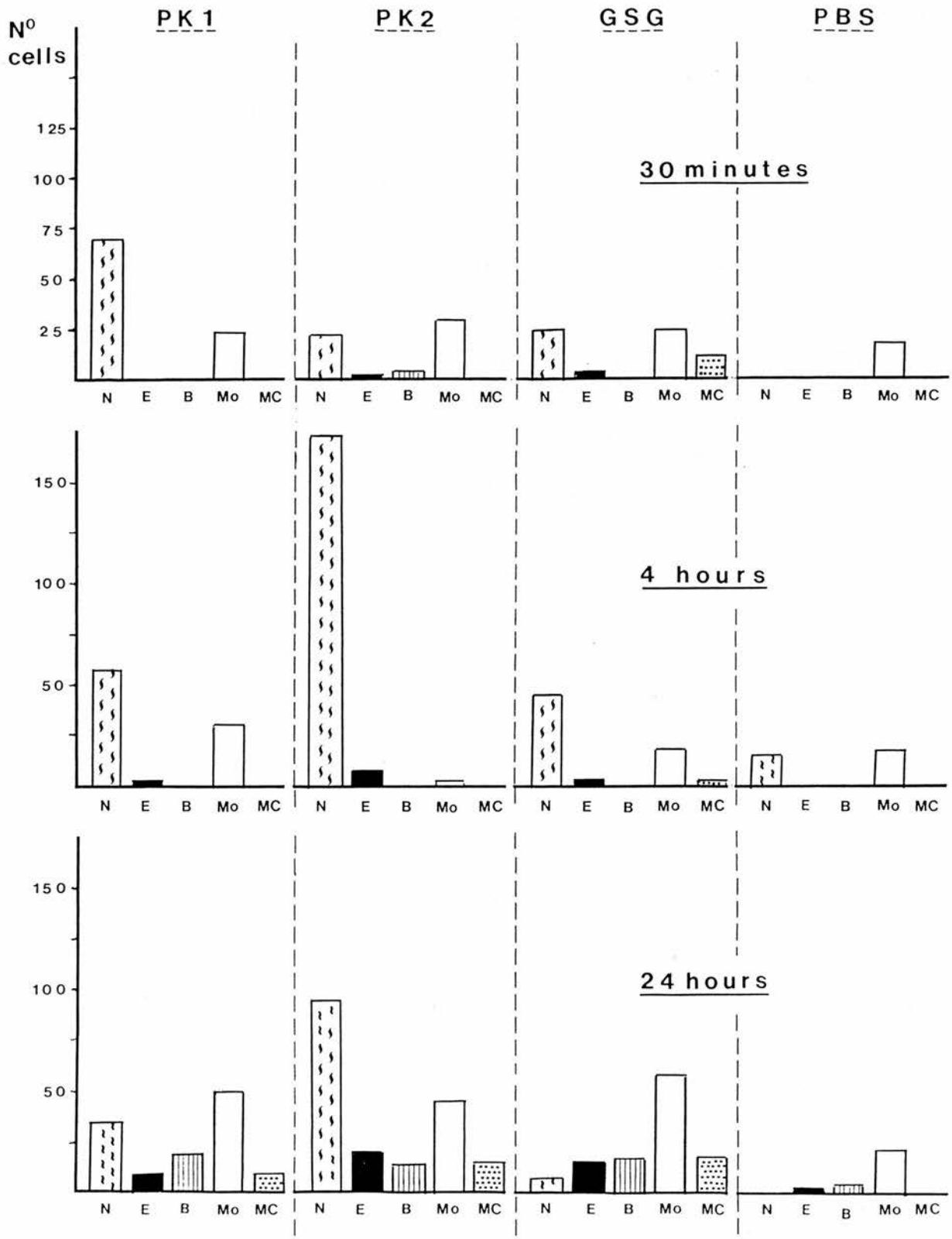
biopsies were taken by three different observers (the author, the supervisor and a collaborator), and results expressed as means are presented in figure 5.10. Comparison of the cellular responses were performed using a two way analysis of variance to test the effect of different antigens and their evolution in time. Due to the nature of the counts, a Kruskal Wallis one-way analysis of variance by ranks (Siegel, 1956) was used to compare responses to the antigens at each time of reading.

At 30 minutes post injection the main feature observed was the infiltration of neutrophils on PK1, PK2 and GSG, with significant higher counts on PK1. Eosinophils were more noticeable on GSG, and basophils on PK2, although these cells were present on all slides except the control and differences between antigens were not significant.

At 4 HPI neutrophils continued to be the most predominant infiltrated cell type. Some neutrophils were also found on PBS. Differences in the responses were significant between PBS and the antigens but also between PK2 and the other two. At this time, more eosinophils were observed on PK2 and mast cells were more abundant on GSG, but differences were not significant.

At 24 HPI the pattern of infiltrated cells became more complex. Neutrophils were the most frequent cell type on PK2. Counts differed significantly between all antigens as well as the control. At this time eosinophils, basophils and mast cells were evident in all reactions displaying significantly higher counts than earlier reactions, but differences in cell counts between the antigens were not so evident. Infiltrated mononuclear cells were significantly more numerous at this time but significant differences between

Figure 5.10. Differential cell counts in skin biopsies taken 30 minutes, 4 hours and 24 hours after the intradermal injection of different antigens isolated from Rhipicephalus appendiculatus ticks in a rabbit immune to the tick. Means for three readings of infiltrated cells in the epidermis are illustrated. Cell codes are as follows: N = neutrophils, E = eosinophils, B = basophils, Mo = mononuclear non resident cells, MC = Mast cells. The antigens used were, two extracts separated from larval homogenates by anion exchange chromatography (PK1 and PK2), a salivary gland homogenate from adult females (GSG), and saline control (PBS). For neutrophils a highly significant effect of antigen type, time and their interaction ($P < 0.0001$), was demonstrated. For eosinophils the same occurred but lower levels of significance were evident (P: antigen = 0.02, time < 0.0001, interaction = 0.03). For basophils, infiltrated mononuclear and mast cells, levels differed significantly only according to time (respectively: $P < 0.0001$, $P < 0.02$ and $P < 0.005$).


N° cells
PK 1
PK 2
GSG
PBS
30 minutes
4 hours
24 hours
125
100
75
50
25
150
N
E
B
Mo
MC

antigens and the control could not be demonstrated.

5.3.3.- Study of the relationship between skin hypersensitivity responses and the level of restriction of the feeding of the ticks on individual rabbits.

The relationship between the hypersensitivity responses to the different antigens injected into the skin of the rabbits, and the capacity of each animal to restrict the feeding process of the ticks was studied using single correlation analysis. A matrix of correlations was prepared comparing for each antigen 11 parameters to measure the reaction of hypersensitivity (using first, both single measurements of the reaction, area and thickness at various times after injection, and then using the compiled parameter) with 4 quantitative indicators of the feeding capacity of the tick on individual rabbits (total adult weight, total nymphal weight, total egg mass and transformed susceptibility).

Table 5.6 shows a summary of the significant correlation coefficients found when examining that relationship, using the single measure parameters. Logarithmic transformation of the parameters of tick feeding success were also analysed. When the area of erythema and induration was used as a measure of the hypersensitivity reaction, significant ($P < 0.05$) relationship was demonstrated between the reactivity to GSG at 24 HPI and the four measures of tick feeding success. However, when the increase in thickness was used to measure the reaction, only the correlation with total nymphal weight was significant. Logarithmic transformations of the tick feeding success variables for

Table 5.6. Correlation coefficients of the relationship between parameters of skin hypersensitivity to tick-derived proteins and parameters of tick feeding success, measured on rabbits with different levels of resistance to Rhipicephalus appendiculatus ticks.

	TICK FEEDING SUCCESS			
	Total adult weight.1	Total nymphal weight.1	Total egg mass.1	Transformed susceptibility.2
Area of erythema GSG at 4 h.	-0.47 (-0.29)	-0.60* (-0.53*)	-0.47 (-0.24)	-0.47
Area of erythema GSG at 24 h.	-0.57* (-0.50*)	-0.54* (-0.43)	-0.58* (-0.53*)	-0.64**
Increase in skin thickness GSG at 24 h.	-0.41 (-0.26)	-0.52* (-0.47)	-0.41 (-0.26)	-0.47
Increase in skin thickness PK1 at 48 h.	-0.43 (-0.41)	-0.47 (-0.55*)	-0.44 (-0.43)	-0.38
Increase in skin thickness PK2 at 48 h.	-0.37 (-0.31)	-0.49* (-0.51*)	-0.40 (-0.27)	-0.35
Increase in skin thickness PK3 at 24 h.	0.37 (0.49*)	0.45 (0.40)	0.43 (0.55*)	0.52*

1. Values inside brackets denote correlation coeficients calculated using the logaritmic (log 10) transformation of the tick feeding success parameters.

2. Transformed Susceptibility = $(1 - x)^{0.25}$, where: x = percentage resistance expressed as proportion of one. Control resistance = 0.00001.

* = Significant ($p < 0.05$) correlation coefficient.

** = Highly significant ($p < 0.01$) correlation coefficient

correlation analysis did not help, in general, to enhance the coefficient. Note the values obtained when the relationship with the increase in thickness to PK3 at 24 HPI was examined. A positive correlation coefficient means increased susceptibility to ticks related with increased reactivity. Although this sort of relationship is not expected this fact will be discussed later.

On the other hand, total nymphal weight appears to be a proper candidate to measure the capacity of rabbits to control ticks. This parameter was significantly associated with the reactions to GSG at 24 HPI previously discussed and to the area of erythema at 4 HPI to GSG and the increase in skin thickness to the larval protein PK2 at 48 HPI.

A similar pattern was found when the relationship between the compiled parameters of the hypersensitivity reactions and those used to measure resistance were studied. Results are summarized in table 5.7. The compiled reaction to GSG at 24 HPI was correlated at a significant level with all the parameters used to measure tick feeding success.

Multiple regression models were calculated to obtain a predictive equation for the tick feeding success parameters based on recorded responses in the skin test. The aim was to study the percentage of the variation in the parameter for tick feeding that could be explained by the equation as determined by the multiple coefficient of determination (Bailey, 1981), and test the significance of each variable included in the model and of the total regression. Results are displayed in table 5.8.

All the models produced to explain the tick feeding success parameters included as independent variables the skin test responses

Table 5.7. Comparison of correlation coefficients of the relationship between compiled parameters used to measure the reaction of skin hypersensitivity to tick-derived proteins in rabbits and parameters used to measure the feeding performance of Rhipicephalus appendiculatus ticks, fed on the same rabbits, which displayed various levels of resistance to the tick.

	% resistance	Transformed susceptibility	Total adult weight	Total nymphal weight	Total egg mass
Compiled reaction GSG at 4h.	0.43	-0.28	-0.20	-0.41	-0.22
Compiled reaction GSG at 24h.	0.54*	-0.52*	-0.48*	-0.51*	-0.49*
Compiled reaction PK1 at 48h.	0.35	-0.20	-0.10	-0.25	-0.16
Compiled reaction PK2 at 48 h.	0.26	-0.18	-0.16	-0.27	-0.19

* Significant ($P < 0.05$) correlation coefficient

Table 5.8. Multiple regression models fitted with skin test data to explain the variability of parameters that measure the feeding capability of Rhipichephalus appendiculatus ticks on rabbits with differing levels of resistance to the tick. Skin test used a salivary gland homogenate from adults (GSG) and antigens extracted from larvae by chromatography (PK2,PK3,PK4).

Predicted variable*	Independent variables	Estimated value	't' test variable
Total adult weight	constant	3664	
	Area erythema GSG 4h.	-6.22	0.004
	Inc. thickness GSG 4h.	407	0.039
MCD = 71%	Inc. thickness PK2 24h.	300	0.039
P = 0.0051	Inc. thickness PK2 24h.	-418	0.003
Total nymphal weight	constant	1315	
	Area erythema GSG 4h.	-1.26	0.034
	Inc. thickness PK2 48h.	-61.79	0.120
MCD = 47%			
P = 0.0163			
Total egg mass	constant	1132	
	Area erythema GSG 4h.	-1.44	0.041
	Inc. thickness PK2 24h.	183	0.016
MCD = 57%	Inc. thickness PK2 48h.	-178	0.012
P = 0.0150			
Transformed susceptibility	constant	0.695	
	Area erythema GSG 24h.	-0.0002	0.003
	Inc. thickness PK3 24h.	0.1756	0.018
MCD = 61%			
P = 0.0013			
Transformed susceptibility	constant	1.0127	
	Area erythema GSG 24h.	-0.0002	0.003
	Area erythema PK1 24h.	-0.0013	0.008
MCD = 83%	Inc. thickness PK3 24h.	0.2580	0.003
P = 0.0008	Inc. thickness PK4 4h.	0.1477	0.206

* MCD = Multiple coefficient of determination of regression.
P = Probability value for null hypothesis for regression.

to GSG at 4 HPI and to PK2 at 48 HPI. However, the multiple coefficients of determination were not too high. Two different models to explain the parameter transformed susceptibility are presented. These included as predictor variables the area of reaction to GSG at 24 HPI and the increase in thickness to PK3 at 24 HPI. The compiled parameters of skin test results showed low levels of significance when included in the models.

5.3.4.- Antibody levels to the salivary gland homogenate, and their relationship with tick resistance and with the responses in the skin test

Antibody levels to GSG observed in the sera of fifteen of the rabbits two weeks after the challenge infestation were used to study the relationship between development of antibody titres after challenge and reactivity in the skin test. Their relationship with resistance (3.6.4) was also examined.

Antibody levels ranged from 2.3 to 4.8. No significant differences were demonstrated when the means of the various groups of rabbits were compared. Observed means were: Gr1(3 instars) = 4.1; Gr2(larvae) = 3.3; Gr3(nymphs) = 4.1; Gr4(adults) = 3.4 and Gr5(control) = 2.7. However, significant differences ($P = 0.03$) were displayed when levels in the controls (only subjected to the challenge infestation), were compared with those repeatedly exposed to the tick (mean = 3.7).

When the relationships of antibody titre and skin test results were examined, significant positive correlations were demonstrated with the parameters total reaction to GSG at 24 HPI ($r = 0.58$. $P <$

0.05) and with total reaction to PK2 at 48 HPI ($r = 0.53$, $P < 0.05$). On the other hand, a significant negative correlation was found between antibody titre and total nymphal weight ($r = -0.51$, $P < 0.05$) and the relationship with the transformed susceptibility was almost significant ($r = -0.48$, $P < 0.1$). However, when data of antibody titre was included on the multiple regression models, low levels of significance were obtained.

5.4.- DISCUSSION.

All the rabbits exposed to ticks acquired resistance expressed as an impaired feeding capacity of the tick when compared with that of ticks feeding on naive animals, but no difference was noted in the degree of resistance in the groups of rabbits with various types of exposure to the tick. This suggests that tick numbers of different instars used during the immunization phase were equal, and that the antigens injected by different instars were homologous. Tick numbers used in this experiment were similar to those used by Walker, Fletcher and Todd (1989). However, the group of rabbits exposed only to larvae tended to produce higher values on the parameters used to measure the tick feeding success, and this group also showed lower reactivity on the skin test and lower antibody titres, indicating that the cross resistance was not complete.

The compiled parameter used to measure resistance, appears to be an adequate way to compare these results with the resistance developed to a one-host tick like B. microplus, although larvae were not included in the challenge infestation. On the other hand, the mixed parameter hides the differences in resistance to various

instars. The parameter did not take into account the individual differences in the non immune restriction of feeding on the control rabbits, because all controls were given a value of 0.00001. The exponential transformation of the resistance was shown to be an effective way to normalize the data.

The parameter total nymphal weight, showed better correlation with the results of the skin test and of the ELISA than the other two parameters to measure tick feeding success. These findings seem to be associated with the better discrimination between naive and resistant animals obtained with the total nymphal weight values, as demonstrated in figure 5.3. This is in agreement with the results of Walker, Fletcher and Todd (1989).

Cutaneous responses to the injected antigens showed peak reactivity at 24 HPI for GSG and at 48 HPI for the larval antigens PK1 and PK2. Cellular changes in the dermis of an immune rabbit demonstrated early infiltration of neutrophils, incipient at 30 minutes but very marked at 4 HPI, followed by recruitment of mononuclears, basophils and eosinophils at 24 HPI. Reactions to all materials resemble a cutaneous basophil hypersensitivity (Roitt, Brostoff and Male, 1985), due to the late appearance of basophils and mononuclears. However, the early presence of neutrophils is indicative of underlying reactions, that might include the activation of complement or be the result of pharmacologically active components, as has been suggested for salivary components of ticks (Gill and Walker, 1985; Walker and Fletcher, 1986). This non immune reaction was stronger at the sites of injection of the larval derived antigens.

No previous reports have been made on skin hypersensitivity to

R. appendiculatus larval proteins in resistant rabbits, but these findings are similar to those of Wikel, Graham and Allen (1978), using the Dermacentor andersoni - guinea pig model and a salivary gland extract. They described a slight immediate skin reactivity caused by a vasoactive component, followed by a delayed cell mediated immune response. On the other hand, Binta and Cunningham (1984) studied the hypersensitivity responses on calves to a R. appendiculatus larval isolate very similar to that used in this work and described immediate hypersensitivity responses associated with the previous exposure of the calves to the tick.

The compiled parameter of area and thickness proved useful to interpret the hypersensitivity reaction, but the relative sensitivity of each single parameter to measure different types of responses (such as, area to measure oedema and erythema, and the thickness to measure induration) could be lost.

The size of skin hypersensitivity reactions to GSG at 24 HPI, showed a good degree of correlation with the measures of resistance. A purification of the proteins contained in this material to reduce unspecific non immune reactions could produce a more useful tool for discrimination of resistant animals. The high positive correlation found with the antigen PK3, could indicate higher cell infiltrates in the skin as a response to a higher sensitivity of the animals to non immune chemotactic factors contained in the extract. The tick would use this susceptibility of the animal as an aid to feeding.

Results obtained on the multiple regression models demonstrate the complex interaction of the skin responses to different antigens and their relationship with resistance. The presence of reactions to

GSG on all the significant models indicates the close relationship between the resistance to the tick and the hypersensitivity responses to this antigen. However, the importance of reactions to the larval antigens could not be ruled out entirely, mainly for the antigen PK2.

Wide variability in the skin responses were found in the experimental animals, even in the controls, and this could help to explain the disparity of the results. In order to understand this, it must be remembered that each of the antigens used in this experiment was a complex mixture of proteins which induced overlapping non specific inflamatory and immune reactions, a fact that is common in tick-derived materials (Willadsen, 1980a). For this reason due to the limited number of experimental animals and to the nature of the laboratory model, direct extrapolation of this methods for work in cattle is not advisable.

5.5.- CONCLUSIONS

- Rabbits acquired resistance to the tick R. appendiculatus after the repeated feeding of any instar on them, and their levels of resistance were well different from those of naive animals.
- Hypersensitivity responses to the different antigens in the skin of the rabbits were of a delayed nature, showing peak reactivity at 24 hours for the salivary gland homogenate and at 48 hours for the larval antigens. These responses are very different to those observed in cattle. The observed reactions were the result of a complex interaction of immune responses and

of the activity of non specific mediators of inflammation contained in the extracts.

- Skin responses to the salivary gland homogenate were shown to be correlated with resistance, but the non specific responses observed in the controls indicate that further purification of such material is advisable for use in a skin test. The interaction with the responses to the larval antigens demonstrates that they must be included in the design of further experiments.

CHAPTER SIX

PRELIMINARY EVALUATION OF THE RESPONSES INDUCED BY THE INOCULATION OF LARVAL Boophilus microplus ANTIGENS PURIFIED BY ANION-EXCHANGE CHROMATOGRAPHY.

SUMMARY

A group of Holstein calves and a group of crossbreed Criollo cattle with previous exposure to the tick Boophilus microplus were tested for their responses to the intradermal inoculation of larval derived antigens and the relationships between these responses and the resistance of the animals to the tick were examined. Observed responses were of immediate development and all had disappeared 24 hours after the inoculation.

Significantly higher responses in the skin test as well as higher levels of resistance were found in the group of Criollo cattle. A strong relationship was demonstrated between the responses to one of the antigens at four hours post injection and the measure of resistance to the tick, but a second test based only on Holstein calves did not replicate those findings. Factors affecting the replicability of the tests under field conditions are discussed.

6.1.- INTRODUCTION

In chapter five, it was seen that antigens purified from larval Rhipicephalus appendiculatus ticks elicited delayed hypersensitivity responses in the skin of rabbits exposed to the tick, and various degrees of correlation between the size of the reactions and resistance to the tick were demonstrated. On the other hand, in

chapter four it was explained that during the preliminary period of study at the CTVM and using the same techniques followed with R.appendiculatus extracts, anion-exchange chromatography was performed on B. microplus larval materials. The purified peaks of protein were freeze dried and transported to Colombia, to conduct field experiments in the development of the skin test for resistance.

These preliminary observations were designed in order to gain knowledge on the dynamics of the reactions elicited by the inoculation of those extracts into the skin of animals with previous exposure to ticks, and to make preliminary observations on the relationship between the skin test responses and the resistance of the animals to the tick, using animals readily available at the research station La Libertad, located in the tick infested area.

6.2.- MATERIALS AND METHODS

6.2.1.- Experimental design and chronology of the skin tests.

A first test designed to study the dynamics of the reactions elicited in the skin of tick exposed animals was conducted during December 1986 to February 1987, on 26 animals using 9 testing sites per animal. Two groups of animals were included (see experimental animals). A second test was conducted between the end of April and begining of May 1987, to study the reactivity to different doses of the antigens. This time only 12 of the above mentioned animals were used, with 17 testing sites per animal. Between six to eight animals were tested on each visit.

6.2.2.- Experimental animals

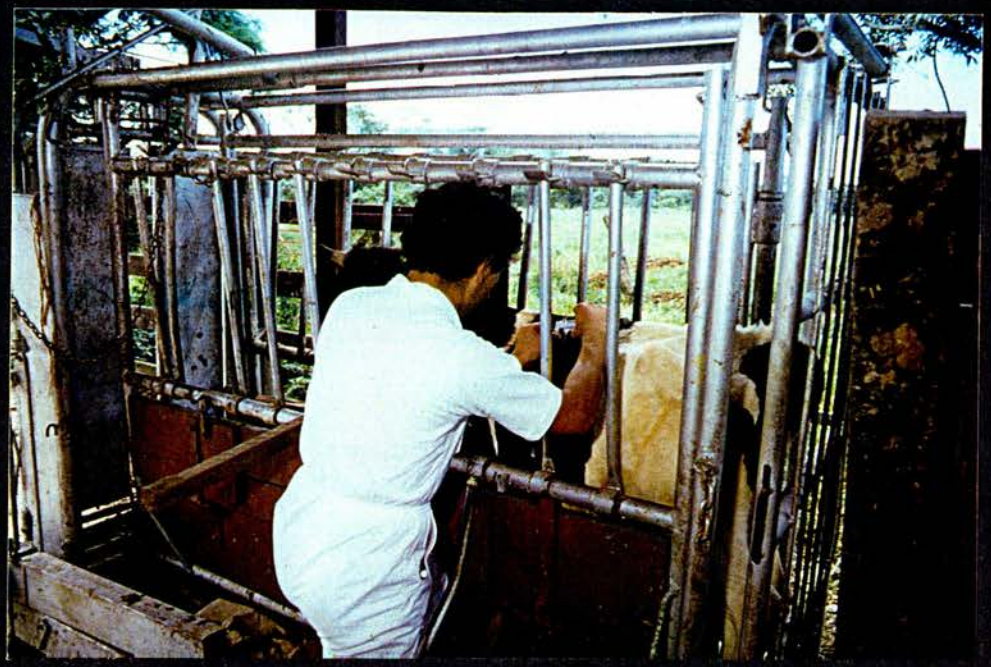
A group of 21 yearling Holstein calves, born in the tick free area but brought to the experimental station when three months old and heavily exposed to B. microplus ticks during previous experiments on tick borne diseases, were used and designated as group one during the first test. Group two for this test consisted of 5 crossbreed Criollo animals (including some zebu blood) that had been used in an experiment of seasonal fluctuations of ticks (Benavides, Villar and Gonzalez, 1988) and had been run without tick control measures since 1983. All animals of group two were more than two years old. For the second test 12 animals of group one were chosen at random.

All animals were grazed in the same paddock consisting of a pasture of Brachiaria decumbens that did not have a heavy burden of B. microplus larvae in the pasture. No other species of ticks were observed on the animals.

6.2.3.- Skin tests

A similar procedure to that described for use on rabbits in the previous chapter (5.2.4), was followed for the skin test. Preliminary trials were performed on the side of the neck, but it was impractical for work in the cattle crush with older and fractious animals. For this reason, the area over the back of the rib cage was used for the test (figure 6.1). All tests used a standard ammount of 0.1 ml of the solutions, injected intradermally with a disposable tuberculin syringe and 26 gauge needle. Testing

Figure 6.1. Performance of the skin test on the calves. Note how the use of an area in the back of the animals allow the measurements to be made in the cattle crush without danger for personnel when working with adult fractious animals.



sites for each antigen were assigned at random, and a PBS control was always included.

The first evaluation used the larval antigens separated by chromatography at the CTVM described in chapter four (4.3.2.1). These freeze dried materials were diluted before use in 2 ml of PBS. Two different dilutions of a salivary gland homogenate of B. microplus females and a crude larval homogenate, were also used as a reference point in this test. Methods for the preparation of these homogenates are described in chapter three (3.3).

The following were the antigens used with their corresponding concentrations of actual inoculated protein in the 0.1 ml used in the test. The differences in protein concentration in different materials are due to the fact that although they were calculated to be equal on the basis of their protein content before being freeze dried, and of their protein content estimated by the Lowry method after dilution, a more accurate protein estimation method later standardized indicate the presented values. Protein estimation methods have been described (3.2).

- PK1 (larval derived) = 15 µg
- PK2 (larval derived) = 19 µg
- PK3 (larval derived) = 31 µg
- PK4 (larval derived) = 11 µg
- GSG-10 (salivary extract) = 12 µg
- GSG-100 (salivary extract) = 1 µg
- LAR-20 (total larval extract) = 34 µg
- LAR-200 (total larval extract) = 3 µg

Dynamics of the reactions were followed during this experiment by measuring the following parameters; area of reaction (the product of two diameters) at 15 and 30 minutes post injection, and the area and the increase in skin thickness at 4, 24 and 48 HPI (hours post injection).

During the second test, the larval derived materials PK1, PK2, and PK3, and a salivary gland homogenate (GSG) were used. Each antigen was tested at four dilutions (1, 1/10, 1/100, 1/1000). In dilution 1, larval derived materials had the same concentrations of test one and GSG had a protein concentration of 325 µg/ml. This time the area of the reactions at 30 minutes and the area and the increase in skin thickness at four HPI only were recorded.

6.2.4.- Acquisition of data on susceptibility to the tick in the experimental animals.

Data of the first skin test were compared with information on susceptibility to the tick of the animals, which was already available at the laboratory in La Libertad. For the group of Holstein calves, data used for this experiment consisted of the mean of two evaluations based on artificial infestations performed in November and December 1986. Values used for animals of group two were the mean of at least five artificial infestations performed on the animals between January 1984 and July 1986 (Benavides, Villar and Gonzalez, 1988).

For the second skin test, information on susceptibility was obtained using the standard artificial infestation method of Utech, Seifert and Wharton (1978) with the modifications described in chapter 3. The artificial infestation in this group of animals was started on the first of May 1987, using approximately 10,000 larvae (from 0.5 gram of eggs) per calf, of B. microplus ticks from the laboratory colony maintained at La Libertad (chapter three). Tick counts were performed on the 19th, 20th, and 21st May.

6.3.- RESULTS.

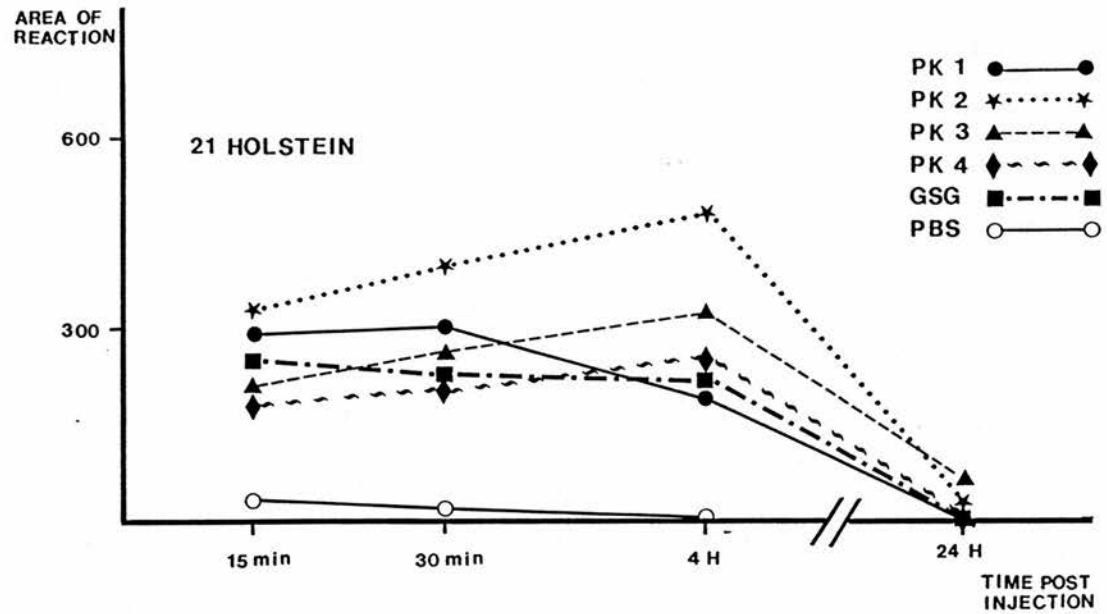
6.3.1.- Dynamics of skin reactions elicited by the antigens.

All the antigens injected into the skin of the animals elicited immediate reactions which disappeared at 24 HPI. For simplicity, responses will be compared between those produced by the antigens purified by chromatography, holding GSG-10 as reference point (figure 6.2), and those elicited by the crude extracts (figure 6.3). Only data on area of reaction are presented, because they demonstrated more consistent patterns.

Strong differences in the skin responses were observed between the two groups of animals. Reactions in the Holstein group were of moderate size at 30 minutes and tended to maintain the same size at four HPI. In the Criollo group, reactions were more severe with a clear division in two types of reactions. Reactions to PK1 and PK3 tended to increase at 4 HPI, whilst those of the other antigens tended to decrease in size. However, the variances of the data at four HPI were too big and no significant differences between responses to the various antigens were observed at this time when data was subjected to analysis of variance (figure 6.2).

Comparison of the responses between the two groups of animals, for each antigen at 30 minutes and at four HPI were performed using a 't' test (Sokal and Rohlf, 1981). Significant differences in the variances between the two groups were observed in all antigens except PK2, and on those values a Cochrane's approximation was used to calculate levels of significance (Sokal and Rohlf, 1981). The means for the group of Criollo cattle were significantly higher

Figure 6.2. Reactions of oedema and erythema elicited by the intradermal inoculation of different antigens derived from Boophilus microplus in the skin of animals exposed to the tick. Data displayed are the mean area of the reaction in a group of 21 Holstein calves (top graph) and of a group of five adult crossbreed Criollo animals (bottom graph). PK1, PK2, PK3 and PK4 identify antigens extracted from larval homogenates by anion exchange chromatography. GSG denotes an extract from salivary glands of females. PBS = saline control. Analysis of variance within each group for the responses at 30 minutes and at four hours, indicates that in the Holstein group reactions to PK2 were significantly ($P < 0.05$) higher than those to other antigens. No significant differences in the responses to the various antigens were found in the group of Criollo cattle.


AREA OF REACTION
600
300
21 HOLSTEIN
PK 1
PK 2
PK 3
PK 4
GSG
PBS
15 min
30 min
4 H
24 H
TIME POST INJECTION

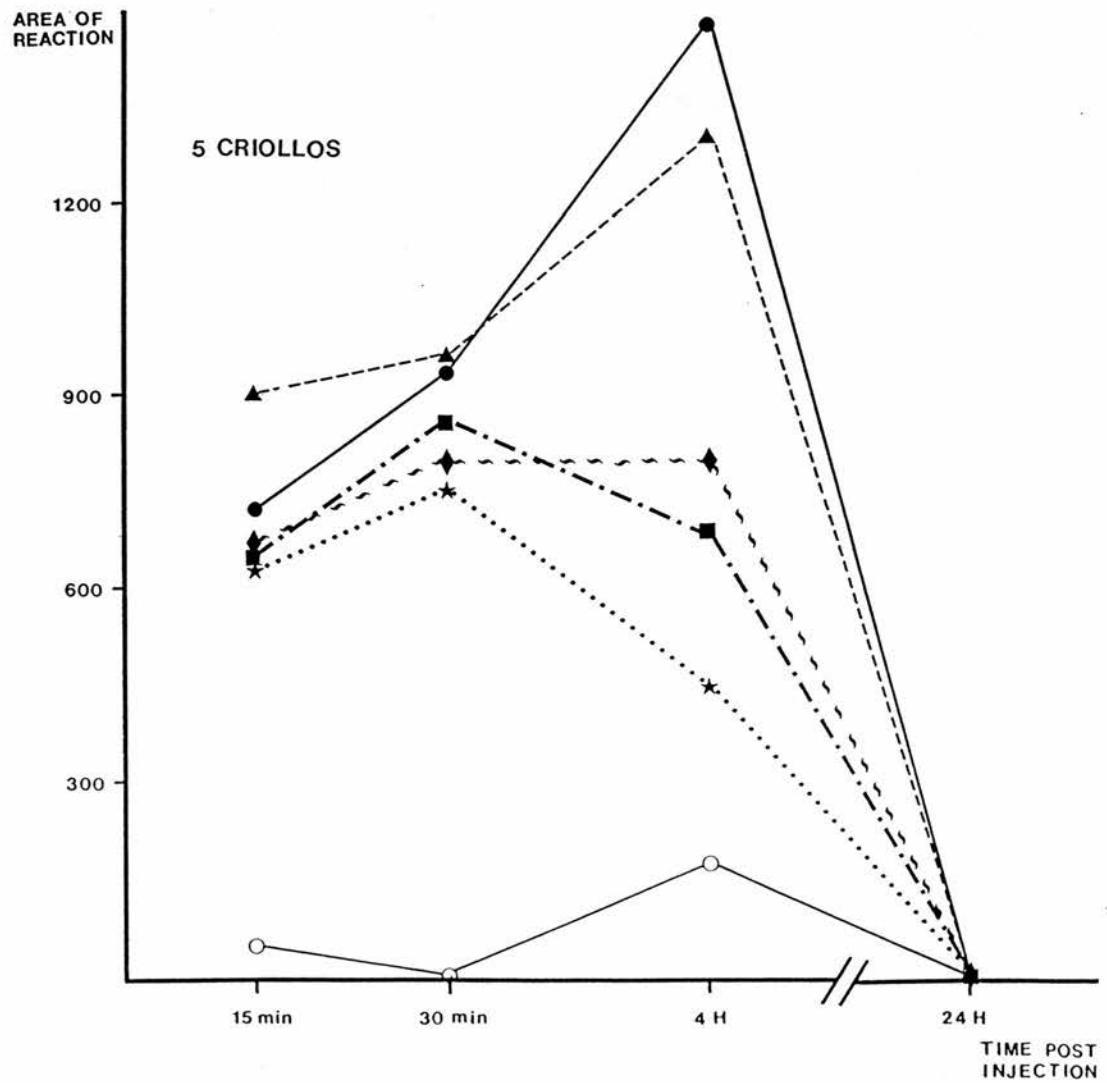
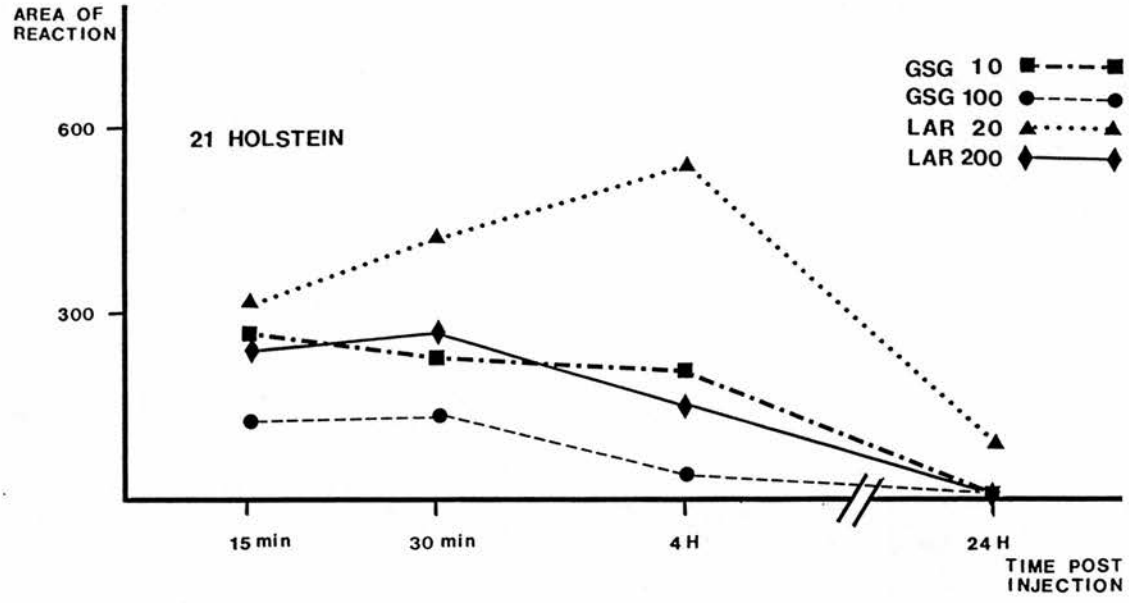
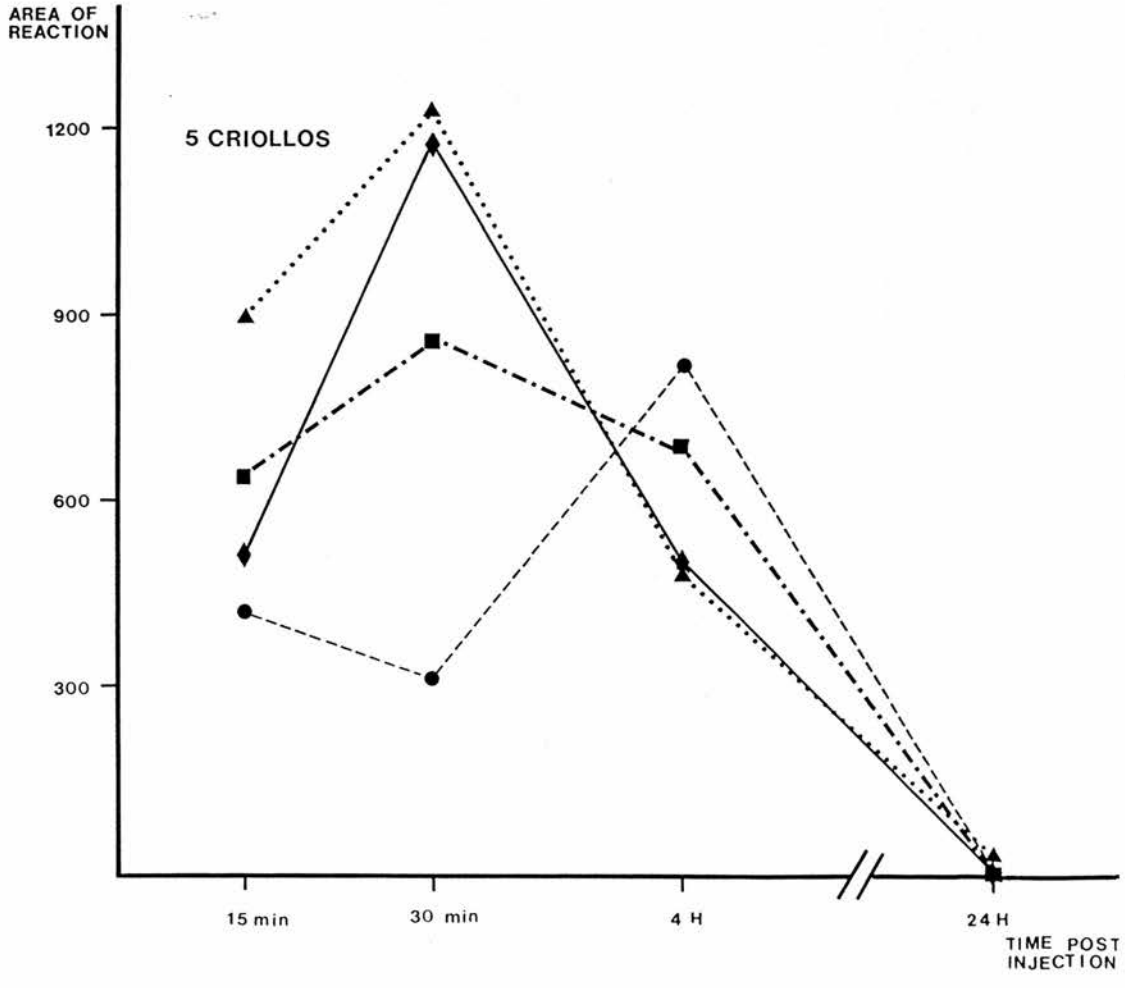

AREA OF REACTION
1200
900
600
300
5 CRIOLLOS
15 min
30 min
4 H
24 H
TIME POST INJECTION

Figure 6.3. Reactivity to crude extracts of Boophilus microplus after the intradermal inoculation of two dilutions of the materials, in groups of animals previously exposed to the tick. The top graph shows mean reactions obtained in a group of Holstein calves. The bottom graph corresponds to the means of a group of 5 adult Criollo crossbreed cattle. GSG-10 and GSG-100 identify respectively inoculated concentrations of 12 and 1 micrograms of protein of a salivary gland homogenate from females. LAR-20 and LAR-200 respectively correspond to inocula of 34 and 3 micrograms of protein of a larval lysate. The Holstein responses to LAR-20 were significantly ($P < 0.05$) higher than to other antigens. In the Criollo group, responses to the larval extracts were significantly higher at 30 minutes post injection, but no differences were found in the reactions at four hours.


AREA OF REACTION
600
300
21 HOLSTEIN
GSG 10
GSG 100
LAR 20
LAR 200
15 min
30 min
4 H
24 H
TIME POST INJECTION


AREA OF REACTION
1200
900
600
300
5 CRIOLLOS
15 min
30 min
4 H
24 H
TIME POST INJECTION

($P < 0.05$) for all antigens except to PK2 and GSG at four HPI.

A similar pattern of reactions for the two groups were found for the crude extracts. The Holstein group showed minute responses at 30 minutes with marked reactions at 4 HPI only for LAR-20 extract. In the Criollo group responses peaked at 30 minutes for extracts LAR-20, LAR-200, and GSG-10. Extract GSG-100 showed peak reactivity at four HPI.

6.3.2.- Relationship of the responses in the skin test and the data on susceptibility to the tick.

Susceptibility to the tick showed a different pattern between the Holstein or the Criollo animals. Values ranged from 7.39% to 28.85% in group one and from 0.03% to 4.8% in group two. (figure 6.4). These data were transformed as described in chapter five (5.3.1) in an attempt to normalize the distribution, but the two groups represented two different populations, since a set of data which is normally distributed are expect to fit a straight line on the rankit plot. This would be the case if data for either group of animals were independently plotted for the transformed parameter. The entire data were used for the correlation analysis because they displayed a good opportunity to assess the capacity of the skin test to predict the susceptibility.

A multiple correlation matrix using the results of the skin test and data on susceptibility to the tick was prepared in the same way as it was described in the previous chapter. Data on susceptibility to the tick were used plain and transformed. Results of the skin test were used as area of the reactions at 30 minutes

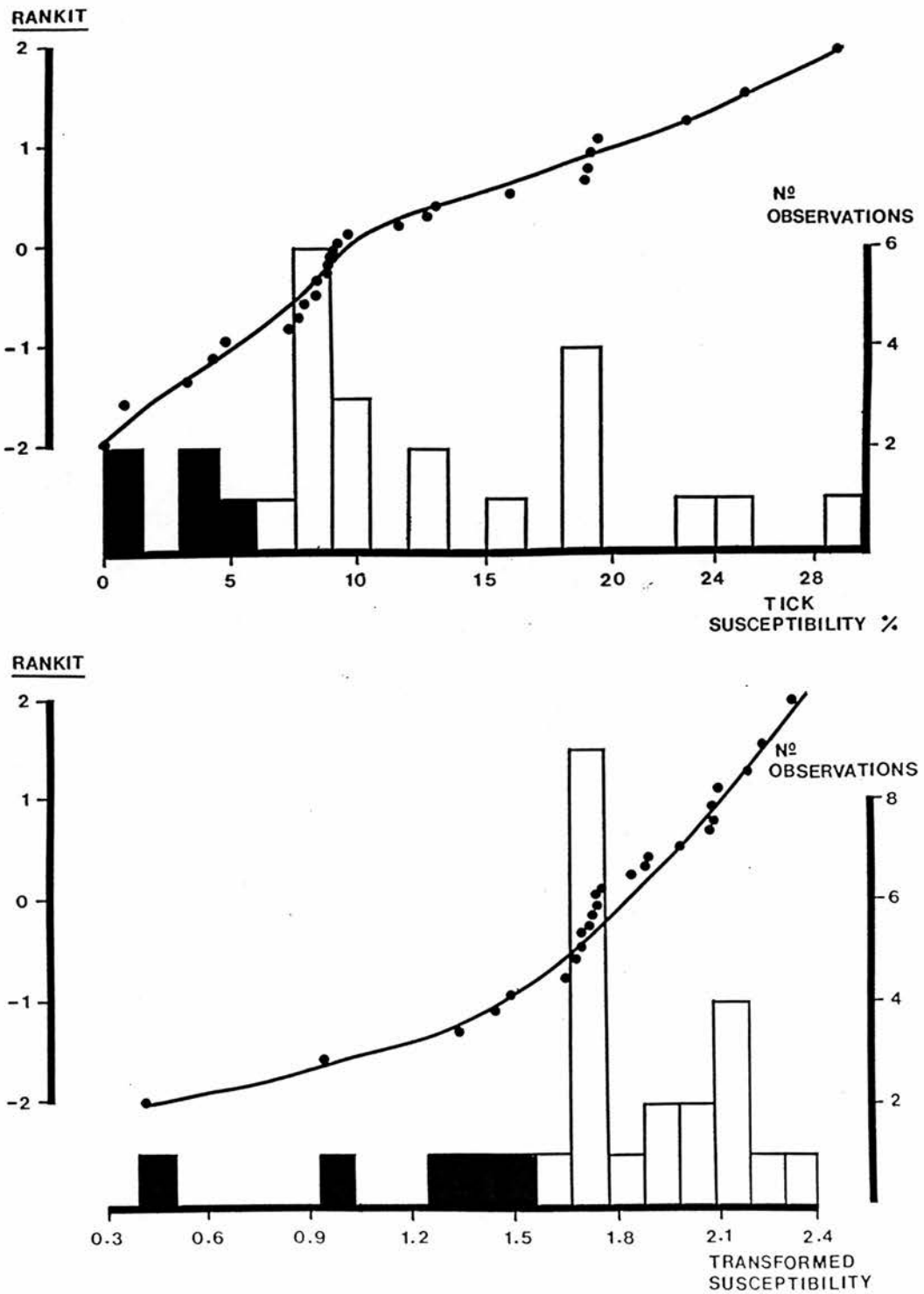


Figure 6.4. Frequency distribution and visual test for normality (based on rankits) on data on susceptibility to the tick Boophilus microplus on 26 animals previously exposed to the tick and maintained under tropical conditions. Shaded bars correspond to the values of 5 crossbreed Criollo adult animals. Clear bars represent values on 21 Holstein calves.

post injection and as area, increase in thickness and total reactions (the product of area and increase in thickness) at four HPI. The more relevant correlations are presented in table 6.1.

Note how the best relationship was found with the antigen PK1. However, significant correlations were also found with various parameters to measure the reaction to PK3, GSG in both dilutions and the LAR-20 antigen. The use of the transformed parameter of susceptibility to ticks showed to improve the measure of the relationship. This can be explained by the visual plot of the variables. Figure 6.5 shows the scattergram of the distributions of the variables oedema to PK3 at 15 minutes and increase in thickness to GSG at four HPI. Although the exponential transformation was not able to linearize the distribution on the rankit plot (figure 6.4), the grouping of the data afforded by the exponential transformation facilitated the measure of the relationship.

6.3.3.- Effect of different doses of the antigens on the skin test responses and on their correlation with tick susceptibility, measured in a group of 12 Holstein calves.

The responses in the skin test were quoted as the area of oedema at 30 minutes and the total reaction at four HPI. For each antigen and each parameter a linear regression of the reactions produced for each concentration of the antigens was calculated. The antigen concentration was expressed as the logarithm of the reciprocal of the dilution used. Figure 6.6 displays a compilation of the results. All regressions were by themselves significant ($P < 0.05$) indicating a clear effect of amount of injected antigen on the

Table 6.1.- Correlation coefficients of the relationship between susceptibility to Boophilus microplus ticks using plain and transformed data, and responses in a skin test using different antigens derived from the tick in 26 animals previously exposed to the tick and grazed under tropical conditions (21 Holstein and 5 Criollo cattle).

			CORRELATION COEFFICIENTS	
ANTIGEN @	time after injection	parameter	mean susceptibility	transformed susceptibility
PK1	30 minutes	Area	-0.39*	-0.67***
	4 hours	Area	-0.48*	-0.77***
		Inc. thickness	-0.70***	-0.88***
		Total reaction	-0.45*	-0.77***
PK3	15 minutes	Area	-0.49**	-0.78***
	30 minutes	Area	-0.46*	-0.71***
	4 hours	Total reaction	-0.45*	-0.71***
GSG-10	15 minutes	Area	-0.50**	-0.68***
	4 hours	Inc. thickness	-0.61***	-0.76***
GSG-100	15 minutes	Area	-0.41*	-0.70***
	4 hours	Area	-0.47*	-0.70***
LAR-200	30 minutes	Area	-0.42*	-0.72***

@= PK1, PK3 : Larval derived antigens separated by chromatography;
GSG-10, GSG-100 : salivary gland homogenate from females in two dilutions;
LAR-200 : crude larval extract.

* = $P < 0.05$
** = $P < 0.01$
*** = $P < 0.001$

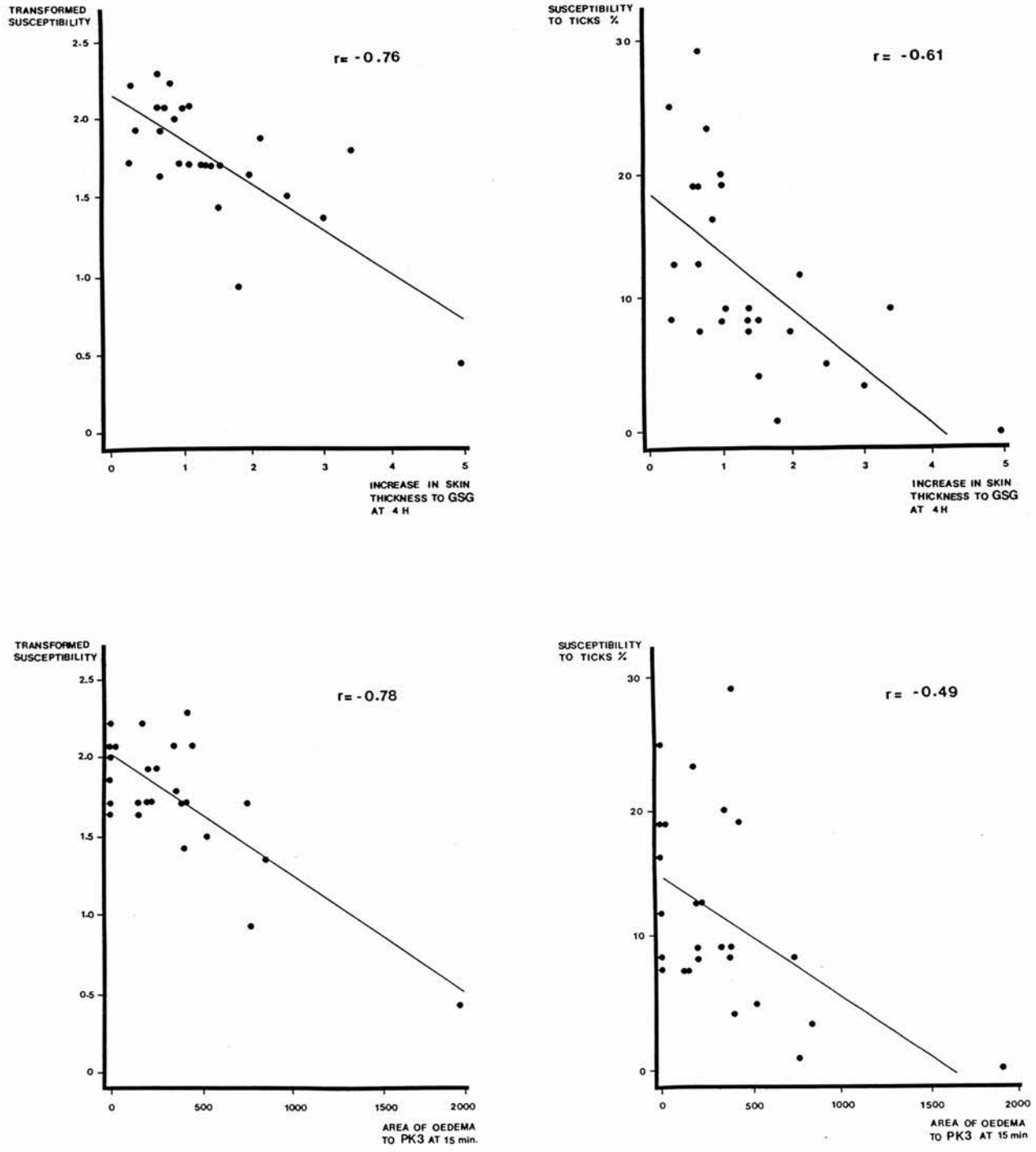


Figure 6.5. Joint distribution of measures of susceptibility to the tick Boophilus microplus (plain and exponentialy transformed data) and the reactions elicited by an antigen extracted from tick larvae by chromatography (PK3) or by a homogenate of salivary glands from females(GSG), in a group of 26 animals with previous exposure to the tick and various degrees of resistance. r = correlation coefficient.

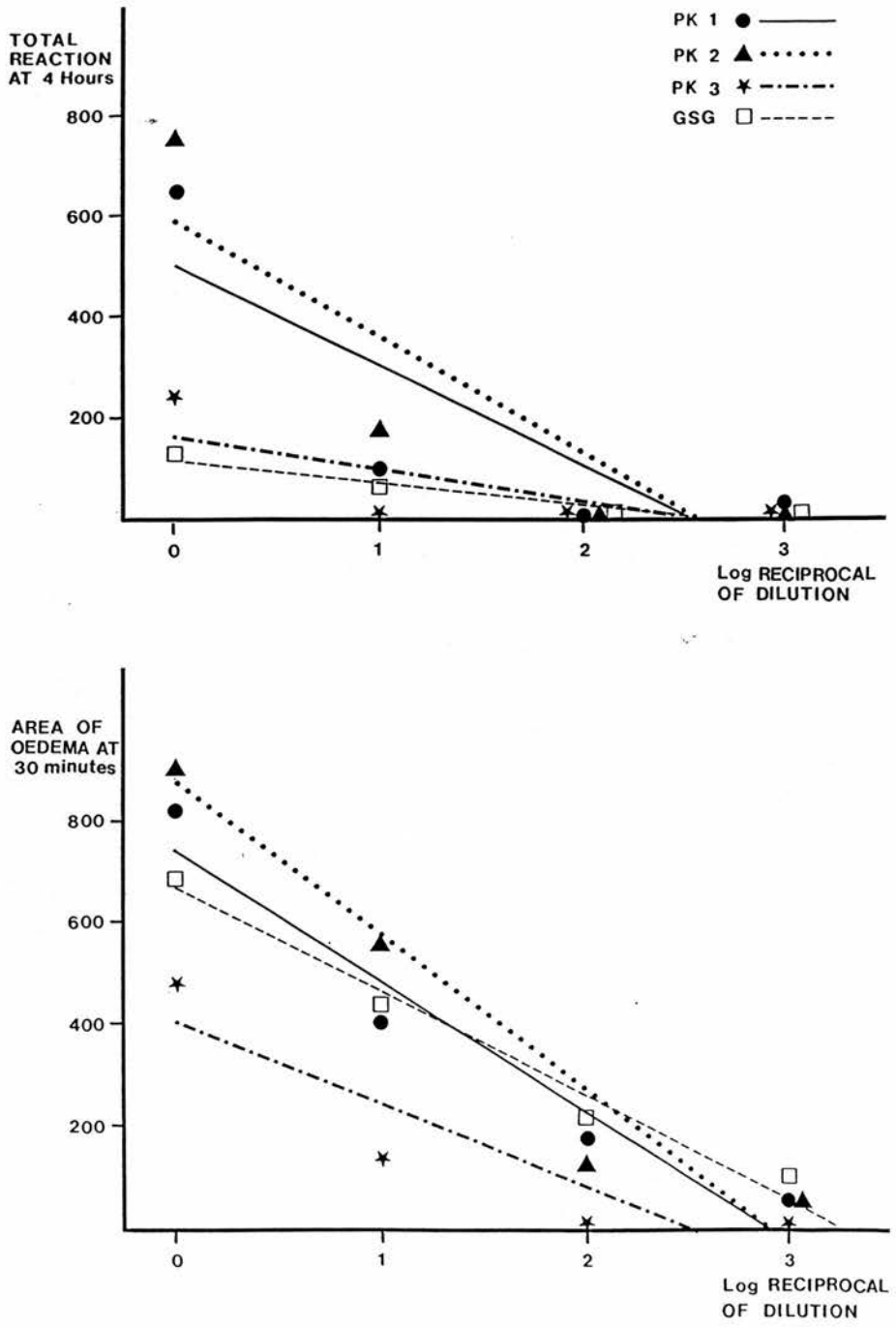


Figure 6.6. Relationship between the size of the elicited reaction and the dilution of the antigen when different concentrations of tick derived antigens were injected in the skin of 12 Holstein calves with wide previous exposure to the tick. A regression line was calculated for each antigen on each parameter and all of them were significant. A variance analysis of the reactions to different antigens for each parameter indicated that although a combined regression was significant, they differed in the slope ($P < 0.05$). Antigen dilution is expressed as the logarithm of the reciprocal of the dilution used.

size of the elicited reaction. However, a variance analysis of the regressions on each parameter (Armitage, 1971) indicated that they differed in the slopes suggesting that it was not the same type of reaction to all the antigens.

Susceptibility to the tick in this group of calves ranged from 2.7 to 8.94, and this time no significant correlation was found between the results of the skin test and the measurement of susceptibility to the tick, neither to the plain nor the transformed data. The relationship between the results obtained during the first and the second evaluations of the skin test and on the assessment of susceptibility to the tick were analysed by a paired 't' test between values obtained for each animal followed by a Spearman's rank correlation test to determine the differences in rank in which each animal was located on each evaluation (Sokal and Rohlf, 1981). Results are displayed in table 6.2. Skin responses in the second evaluation were significantly higher and tick susceptibility values were lower than in the first. No agreement was found betweeen the ranking obtained in the two evaluations.

6.4.- DISCUSSION

The good degree of relationship observed during the first evaluation of this experiment between the susceptibility to the tick and the skin responses elicited to the antigen PK1 at 4 HPI is the type of relationship that could make a skin test very practicable for use in the field. However, the lack of agreement between the two evaluations illustrates that this is not a straightforward procedure. This discussion will emphasize those aspects which could

Table 6.2.- Comparison of parameters of susceptibility to the tick Boophilus microplus and of results in a skin test using larval derived proteins obtained in two evaluations performed on a group of 12 Holstein calves previously exposed to the tick on two occasions (December 1986 and May 1987). Comparisons were performed using a paired 't' test for the differences found in the two evaluations in the same animal, and by a Spearman's correlation of the rank assigned to each animal in each test.

PARAMETER	antigen	't' test		Spearman's rank
		mean difference	't'	coefficient
Susceptibility to ticks		-12.1	5.80 ***	0.021 NS
Area Oedema at 30 minutes	PK1	484.4	3.03 **	-0.627 *
	PK2	368.2	3.40 **	0.174 NS
	PK3	152.2	1.63 NS	-0.130 NS
	GSG	440.2	5.50 ***	0.024 NS
Total reaction at 4 hours	PK1	360.9	0.91 NS	-0.549 NS
	PK2	634.0	0.99 NS	-0.479 NS
	PK3	-377.5	1.31 NS	-0.416 NS
	GSG	-352.4	1.05 NS	-0.090 NS

* = P < 0.05
** = P < 0.01
*** = P < 0.001
NS = no significant

help explain to the variability in the responses.

Tick susceptibility values observed in the Holstein animals during the first evaluation (mean 13.9%), were similar to those reported (14.7%) for that breed (Utech, Wharton and Kerr, 1978). However, a lower mean was observed during the second evaluation (4.6% as compared with the mean obtained in the same animals in the first test 16.7%).

This high susceptibility may have been caused by a stressing factor affecting this group of animals. The dry season that occurs in the area from December to March (Benavides, 1984) is usually associated with nutritional stress on the animals and this affects the level of resistance on the animals (Sutherst et al., 1983b). On the other hand, the animals had previously been subjected to a series of artificial infestations with tick larvae. The immunodepressive effect of tick infestation is well known (O' Kelly, Seebeck and Springell, 1971; Ribeiro et al., 1985) and the animals were kept under an improved management regime from the beginning of January 1987. The lower values of susceptibility obtained during the second test may have been caused by an interaction of better management and the improvement of nutrition that follows the start of the rainy season at the end of April.

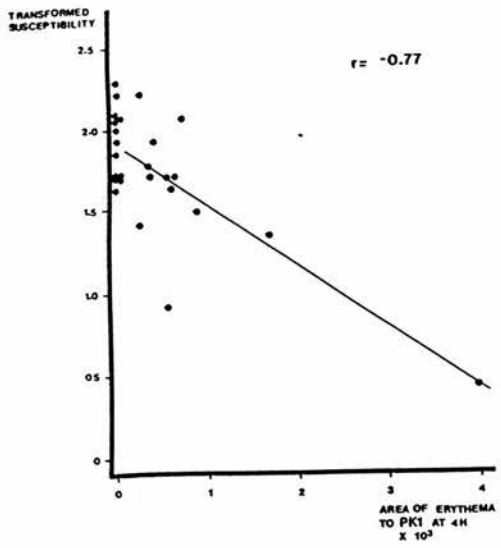
No agreements were found either between the size of the skin reactions obtained during both tests, or in the rank in which animals could be classified on the basis of these results. A visual comparison of the reactions can be made by contrasting reactivity levels at 30 minutes in the Holstein group in figure 6.2 and those of the undiluted materials in figure 6.6. It is clear that reactivity levels were increased during the second test. Stress

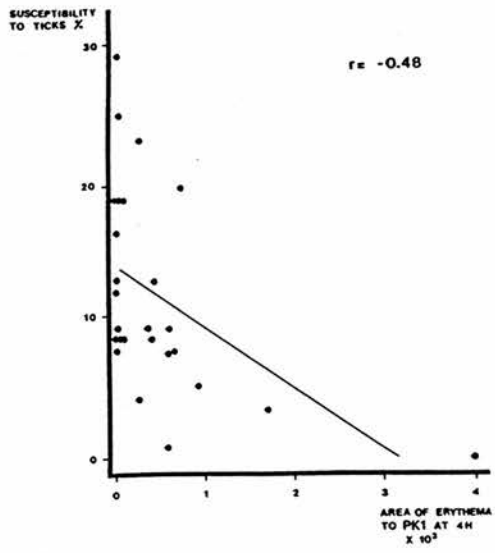
factors also affect the whole immune response (Kelley, 1985) and so this is another reflection of the improved nutrition and management of the animals. However, reactions to antigen PK2 were remarkable since they produced the highest values within the Holstein animals and the lowest within the Criollo group. It is probable that this material contains pharmacologically active materials and that these reactions are not related with immune responses.

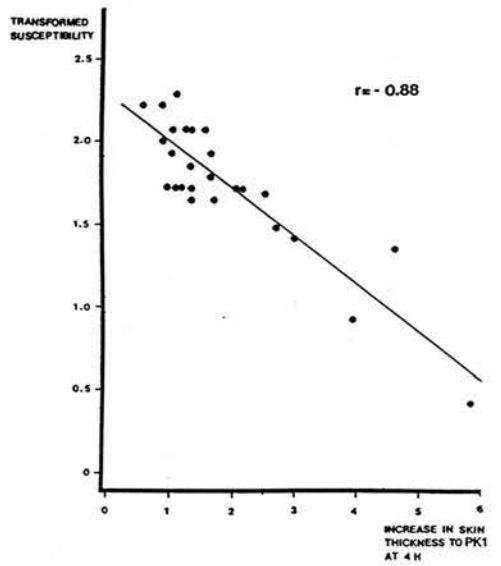
The lack of repeatibility on the measures of relationship between the skin test responses and the susceptibility to the tick are not disappointing, since two facts can help explain these results. To start with, it appears that the type of animals selected for this experiment were not the most appropriate to study this relationship, since they constituted a group in which the values of susceptibility were located on one extreme of the distribution (figure 6.4). They also displayed skin reactions that were located on the lower part of the scale, where they are too difficult to measure accurately (figures 6.5 and 6.7). The inclusion of the Criollo group of animals clearly caused the significance of the correlations.

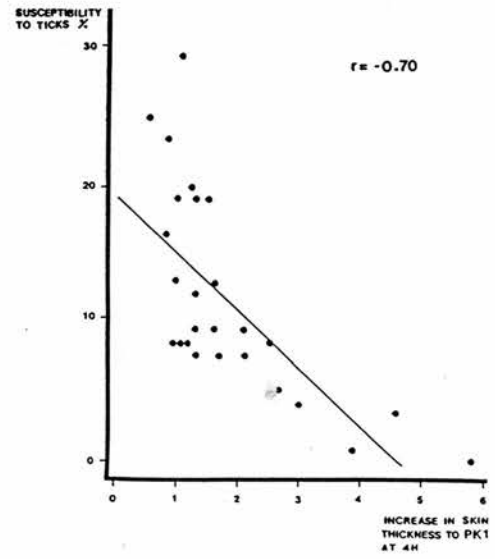
The second point to be clarified is the selection of the measure of the relationship. Correlation analysis implies that both variables are normally distributed (Armitage, 1971) and this is not always easy to obtain with the data of skin test and susceptibility to the tick. As an example of this, figure 6.7 shows the plot of the results obtained for the antigen PK1 at four HPI during the first test, using both groups of animals. The use of the parameter increase in thickness produced the highest coefficients because in

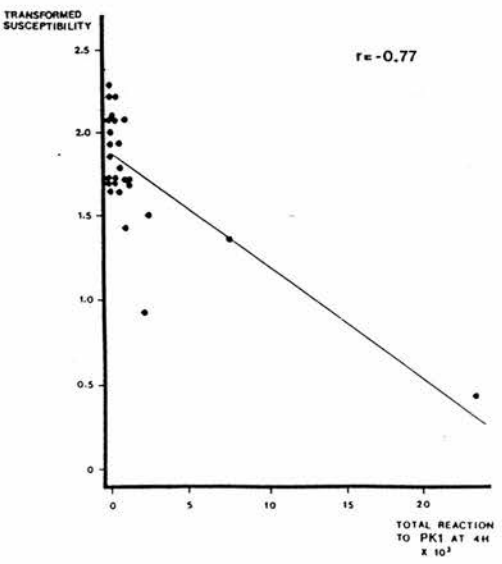
Figure 6.7. Comparison of the effects of the use of different parameters to measure the skin reaction on the correlation coefficient obtained when the susceptibility to the tick Boophilus microplus and the responses elicited four hours after the intradermal injection of the antigen PK1, were studied in a group of animals previously exposed to the tick. The paired data in both parameters of skin test and susceptibility to the tick are presented as dots in the graphic plot. r = correlation coefficient.

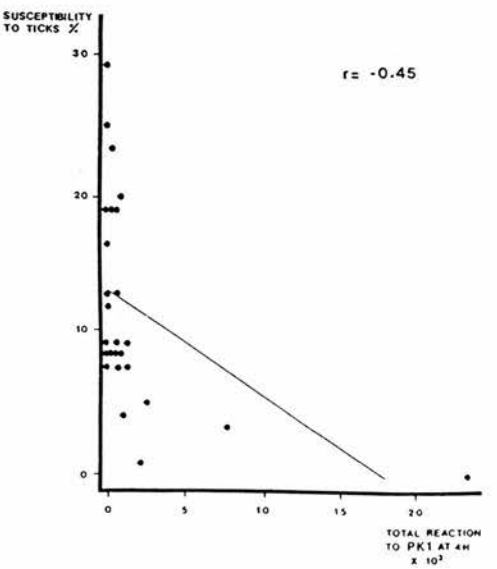

TRANSFORMED SUSCEPTIBILITY
r= -0.77
AREA OF ERYTHEMA TO PK1 AT 4H X 10³


SUSCEPTIBILITY TO TICKS %
r= -0.48
AREA OF ERYTHEMA TO PK1 AT 4H X 10³


TRANSFORMED SUSCEPTIBILITY
r= - 0.88
INCREASE IN SKIN THICKNESS TO PK1 AT 4 H


SUSCEPTIBILITY TO TICKS %
r= -0.70
INCREASE IN SKIN THICKNESS TO PK1 AT 4H


TRANSFORMED SUSCEPTIBILITY
r= -0.77
TOTAL REACTION TO PK1 AT 4H X 10³


SUSCEPTIBILITY TO TICKS %
r= -0.45
TOTAL REACTION TO PK1 AT 4H X 10³

it data tended to be more evenly distributed. Note how the use of the parameters of area or the compiled one, grouped the distribution in the lower part of the scale, and that without the two or three points located at the right of the scale such a high correlation coefficient would not have been obtained.

Data of area of reaction (and also the compiled parameter) tended to increase exponentially, and the reading of the reactions at 4 HPI was associated with subjectivity in delineating the areas. This caused the differences in the responses to various antigens and between the groups of animals to be not significant (figure 6.2). Also, the regression of dose-effect during the second experiment suggest an exponential increase in the responses. It was concluded that a logarithmic or a square root transformation was advisable for this data.

An attempt was performed transforming to square root the responses to the antigen PK1 during the first experiment using the data of the total reaction at 4 HPI. The correlation coefficient was increased by the transformation from - 0.77 to - 0.83 when related to transformed susceptibility and from -0.45 to -0.55 when related to susceptibility. On the other hand, using data from the second experiment, the antigen concentration required to produce a reaction of a size of 500 mm^2 or 10 mm^2 was calculated in the way described by Willadsen *et al.*, (1978) and the values obtained were correlated with data on susceptibility without any obvious improvement of the measure of the relationship.

6.5.- CONCLUSIONS

- Reactions elicited by the intradermal injection of tick derived antigens in the skin of animals exposed to the tick, showed an immediate evolution, with reactions peaking at 30 minutes or four HPI according to the type of antigen. When the antigens were assayed on a group of crossbreed Criollo animals, they displayed significantly stronger reactions than those displayed by a group of Holstein calves.
- Susceptibility to the tick was strongly correlated with the size of the responses in the skin test to the antigen PK1, when measured on the totality of the animals (21 Holstein and 5 Criollos), but such a relationship could not be demonstrated when measured some months later on a group of 12 of the Holstein calves.
- It was concluded that managerial factors affected the responses both in the skin test and in the measurement of the susceptibility to the tick on the animals, and that the group of Holstein calves did not provide sufficient variability required to cover all the range of values that could be expected in animals in the field. A standardization of the above mentioned factors and the use of animals capable to develop a wide range of resistance, are strongly advisable when conducting further experiments in this area.

CHAPTER SEVEN:

ACQUISITION OF RESISTANCE TO Boophilus microplus IN YEARLINGS AND ASSOCIATED CHANGES IN THE DERMAL RESPONSES TO TICK ANTIGENS.

SUMMARY

In the search for a proper tool for the selection of animals resistant to the tick Boophilus microplus, a group of crossbred Gyr-Holstein yearlings were repeatedly exposed to tick larvae and repeatedly skin tested to study the acquisition of resistance to the tick, the evolution of the dermal responses elicited by the injection of tick-derived antigens, the development of anti-tick antibodies in serum, and the relationship between these measurements in each individual. All animals acquired strong resistance to the tick, whilst the size of the reactions and the level of antibodies increased as a consequence of the repeated exposure. Significant correlations were observed between the level of susceptibility to the tick and the size of the dermal reactions induced by the tick-derived antigens, between the susceptibility to the tick and the level of antibodies present in the sera of the animals and between the size of the reactions produced and the antibody content. The discussion emphasizes the applicability of these results to field situations.

7.1.- INTRODUCTION.

In the previous chapter (6.5), the dermal responses to tick-derived antigens were studied on animals previously exposed to the

tick, and various degrees of relationship between those responses and the resistance of the animals to the tick were demonstrated. However, those results could not be repeated when some of the animals were tested some months later, suggesting that stressing factors on the animals affected the responses both in the skin test and in the displayed resistance to the tick. The most important of those stressing factors was considered to be the low nutritional status of the animals, such as that which occurs during the dry season in the eastern savannas of Colombia. At the same time it was proposed that similar experiments should be conducted on animals displaying wider ranges of resistance than those observed on Holstein calves.

On the basis of those findings this experiment was designed to study the dermal responses elicited by the injection of tick-derived antigens on animals naive to the tick, the acquisition of resistance in the animals after repeated exposure to the tick, the changes in the skin test reactions and on the level of antibodies to salivary gland antigens of the tick that followed the acquisition of resistance. During the experiment the animals were kept stalled under a high nutritional plane, in order to minimize the effect of nutritional stress on the resistance to the tick or on the reactivity of the skin test. A group of crossbred Gyr-Holstein calves were used since they were expected to produce medium to high levels of resistance to the tick (Utech, Wharton and Kerr, 1978).

This experiment was conducted using the facilities available in the Tibaitatá research station, located on the Bogotá plateau at 2,640 m a.s.l.

7.2.- MATERIALS AND METHODS.

7.2.1.- Experimental animals.

A group of eleven, 8 to 10 month old, Gyr-Holstein calves (6 females, 5 males) were used. These animals were born at Tibaitatá, which is located on the tick free area of the country, and had no previous exposure to ticks. Throughout the experiment they were offered a diet of corn silage ad libitum and a supplement of concentrate (1 Kg/head/daily).

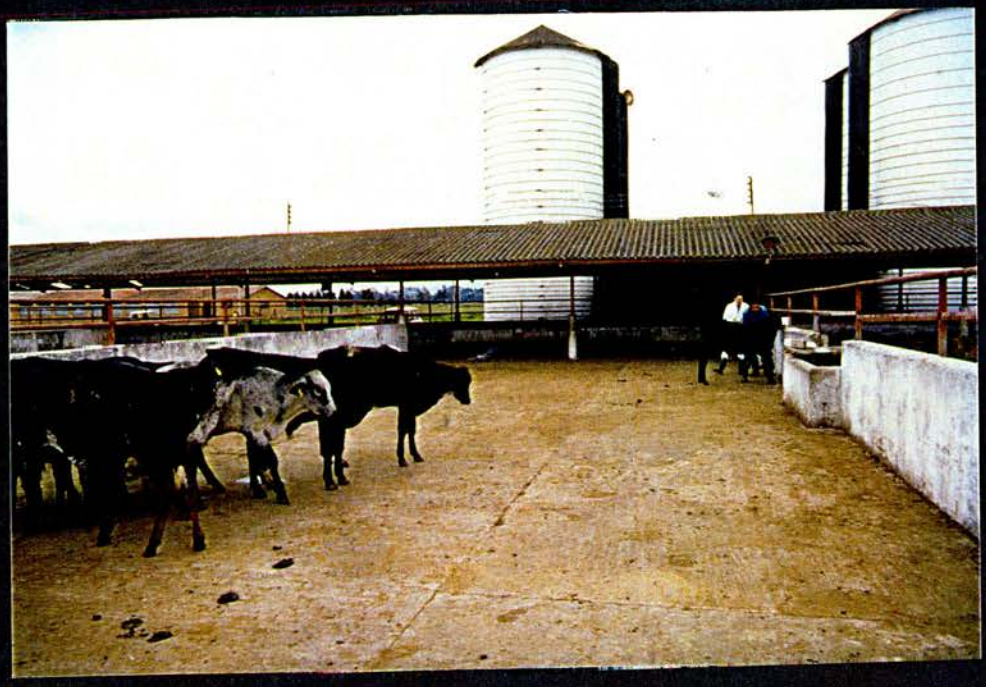
The animals were treated for internal parasites and lice using injectable levamisole (Levamisol, Vecol Colombia) and a coumaphos spray (Asuntol, Bayer Colombia), one month before the start of the experiment and then they were allocated to one of the two different yards: tick free or tick infested (7.2.2).

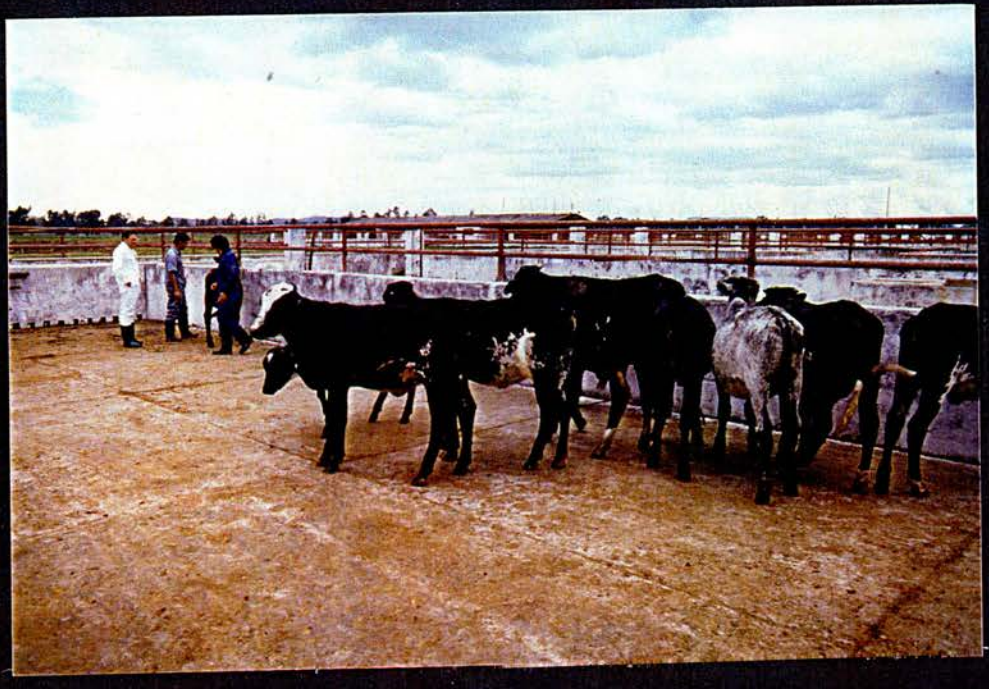
7.2.2.- Experimental design.

Animals were maintained free on two different open, cement floored yards. These yards were designed as tick free or tick infested and were separated by a vacated one. Protective measures were taken around the tick infested yard such as the application of a 20 cms band of grease impregnated with acaricide in order to minimize the risk of escaped ticks. Each yard had an area of approximately 250 m^2 (figure 7.1). As no cattle crush facilities were available the animals were restrained by rope during the different evaluations.

The animals were subjected to repeated skin testing and to

Figure 7.1. Cement floored yard and experimental animals used for the study of acquisition of resistance to Boophilus microplus in yearlings and the associated changes in the dermal responses to tick antigens. Crossbreed Gyr-Holstein calves of both sexes were used in the experiment. To prevent the spread of ticks to nearby cattle of the research station, animals from the tick infested yard were not removed for any reason until the end of the experiment.





repeated exposure to B. microplus larvae using the schedule displayed in figure 7.2. Initially, six animals (3 females and 3 males) were allocated at random to the tick infested yard where they were subjected to the first skin test and tick infestation. From the second test and then so on, a calf from the tick free enclosure was chosen, moved to the tick infested yard and used as control for each infestation. Two controls were used for the final infestation, and controls for the second and third infestation were repeatedly exposed to ticks and skin tested also on the subsequent evaluations. The remaining animal was repeatedly skin tested from the second test and only exposed to ticks on the final infestation. This was the control of repeated skin test.

Under the arrangement described above, data finally collected were: six values with three previous exposures to the tick, seven values with two previous exposures, eight values with one previous exposure to the tick and ten from naive animals, plus the repeated skin test control.

The chronology of the experiment is displayed in table 7.1.

7.2.3.- Measurement of susceptibility to the tick.

Susceptibility to B. microplus ticks was measured using artificial infestations with larvae on day 0 (Wednesday) followed by counts of engorged females larger than 4.5 mm, on days 19 (Monday) to 24 (Saturday) after larvae were applied (Utech, Seifert and Wharton, 1978).

Approximately 10,000 larvae (corresponding to 0.5 grams of eggs) of the haemoparasite free B. microplus colony maintained at

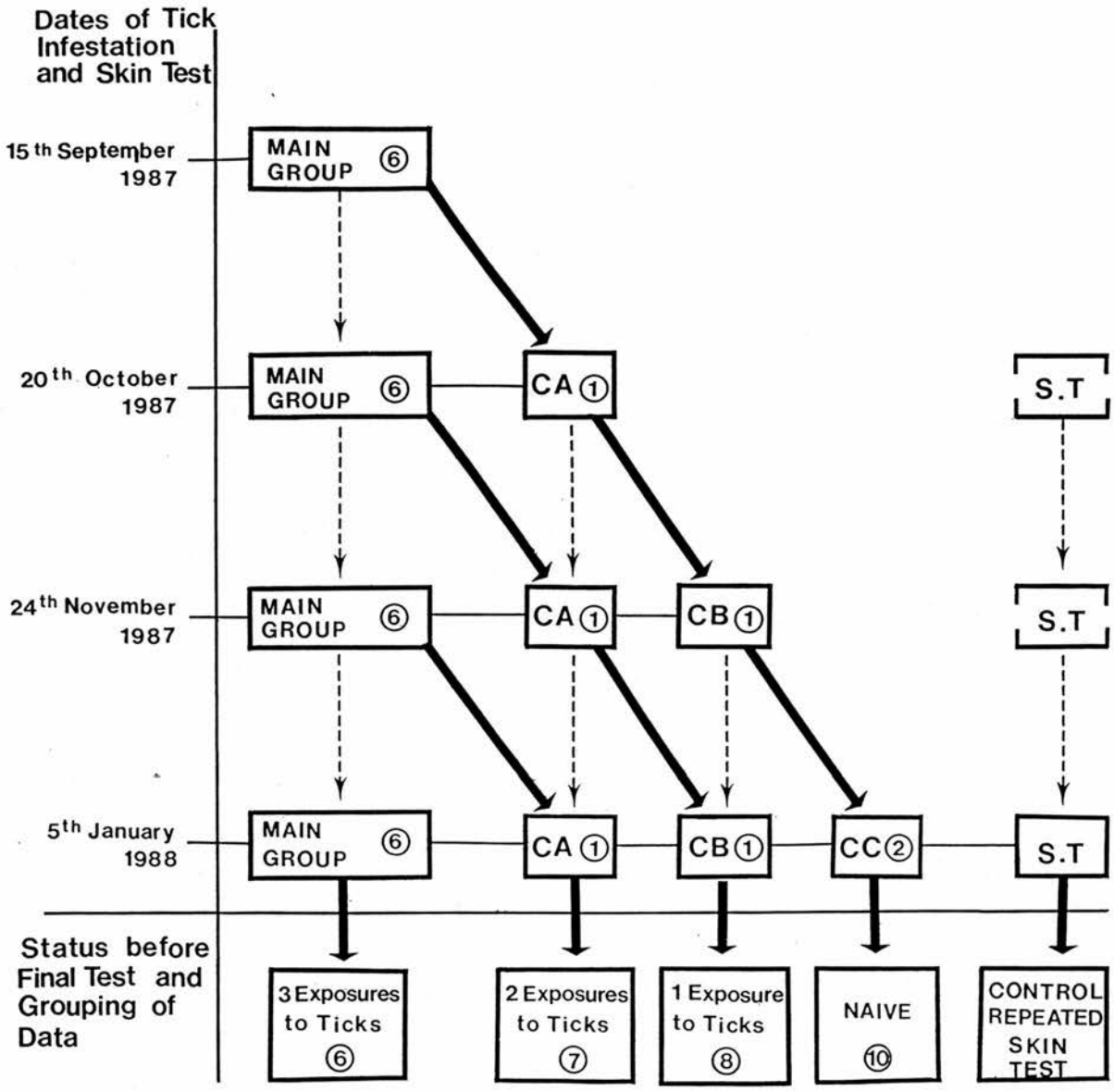


Figure 7.2. Array of the experiment "Acquisition of resistance to Boophilus microplus in yearlings and associated changes in the dermal responses to tick antigens". On each tick infestation a naive control calf was included to be infested and skin tested (CA for second, CB for third and two controls CC for the final infestation). In subsequent tests these calves were also infested and skin tested. The control for repeated skin test (S.T.) was subjected to repeated inoculation of the antigens and only exposed to ticks on the final infestation. The numbers inside circles indicate the number of animals in each group.

Table 7.1. Chronology of the activities related with the experiment: Acquisition of resistance to Boophilus microplus in yearlings and associated changes in the dermal responses to tick antigens.

Date	Activity*	Animals involved in the activity
3/8/87	First collection of sera	11
15/9/87	First intradermal test (M)	6
16/9/87	First tick infestation (M)	6
5-10/10/87	First tick count	6
10/10/87	Application of acaricide	6
20/10/87	2nd collection of sera and 2nd skin test (M+Ca+Cs)	8
21/10/87	2nd tick infestation (M+Ca)	7
9-14/11/87	2nd tick count	7
14/11/87	Application of acaricide	7
24/11/87	Third collection of sera and Third skin test (M+Ca+Cb+Cs)	9
25/11/87	Third tick infestation (M+Ca+Cb)	8
14-19/12/87	Third tick count	8
19/12/87	Application of acaricide	8
5/1/88	4th collection of sera and 4th skin test (M+Ca+Cb+2Cc+Cs)	11
6/1/88	4th tick infestation (M+Ca+Cb+2Cc+Cs)	11
25-30/1/88	4th tick count	11
30/1/88	application of acaricide	11
2/2/88	Final collection of sera	11

* M = animals of main group (6)
Ca = control for 2nd infestation
Cb = control for 3th infestation
2Cc = controls for 4th infestation (2)
Cs = control of repeated skin test

the laboratory (3.1.2) were used to infest each animal (3.1.3). On an infestation day larvae were applied at 8 a.m. and the tubes containig them were left in place for four hours. After the tick count on day 24 the animals were sprayed with a solution of coumaphos to kill any ticks remaining on the animals.

Susceptibility to the tick was calculated as described in chapter three (3.1.3). Percentage values were transformed before the statistical analysis in the same way as described in chapter five (5.3.1).

7.2.4.- Skin tests.

The changes in the reactivity of the animals to tick-derived antigens was studied by repeatedly conducting a skin test on the animals as they were exposed to the tick, using a methodology similar to that described in chapter six (6.2.3). The antigens used in the test were the larval derived: PK1, PK2, and PK3 isolated by chromatography at the CTVM, and a B. microplus salivary gland homogenate (GSG) freshly prepared the day before the test by grinding up 10 salivary glands in 1 ml of PBS, as already described (3.3.2).

Injected antigen concentrations were: PK1 = 5 µg, PK2 = 7 µg, PK3 = 7 µg and GSG = 10 µg. (concentrations used in the previous chapter were reduced due to problems of availability of purified proteins for all the inoculations required in this experiment).

The test was conducted the day before tick larvae were applied, and reactions developed at 30 minutes, 4 and 24 HPI (hours post injection). The diameters in two directions and the increase in skin

thickness of the reaction were measured in the same way as described in chapter six (6.2.3), but the product of both diameters was transformed to square root before the analysis (and quoted as diameter of reaction), and the compiled parameter was calculated multiplying this value with that of the increase in thickness. The normal skin thickness at any injection site (pre-injection value) was also recorded for use in the correlation analysis.

7.2.5.- Determination of antibodies to salivary antigens after repeated exposure of the animals to the tick.

Sera were obtained from the experimental animals at various stages during the experiment: firstly for all the animals, then before any subsequent skin test and one week after the termination of the last feeding cycle (table 7.1). Sera were maintained frozen at -20° C and in November 1988 were transported to Edinburgh, where an ELISA test was conducted on the sera using GSG as antigen.

End point titre values for each sera were calculated on a standard reference curve using the absorbance readings obtained with the sera at a dilution of 1:200. Results are expressed as base 2 logarithm of 1/100 of the reciprocal of the dilution. Methods are fully described in chapter three (3.6).

7.2.6.- Statistical analysis.

For most of the parameters analysed, a regression line was calculated for the evolution of the responses of each individual animal in the repeated tests, and a combined regression was fitted using data from all the animals (Sokal and Rohlf, 1981). An analysis

of variance of the regressions was used to compare the responses between individuals. Responses in the controls where applicable were compared in the same way.

7.3.- RESULTS.

7.3.1.- Acquisition of resistance after repeated exposure to the tick.

Acquisition of resistance was expressed as reductions in the values of susceptibility to the tick. Animals naive to the parasite showed a mean value of 7.8%. A regression line of the values of transformed susceptibility after each exposure was fitted for each animal. Results are presented in figure 7.3, where data and regression are presented in the untransformed scale to display the individual variations. The combined regression for the six animals of the main group and the control for the second infestation was highly significant ($P < 0.01$). The mean values of susceptibility according to the previous exposure to the tick were: 1 exposure = 3.9%, 2 exposures = 1.99% and 3 exposures = 0.89%. No significant differences in the slopes or in the constants were detected between regressions for individual animals.

A tendency for a higher susceptibility was observed after two previous exposures to the tick. Examination of the values obtained from the controls used for each infestation ruled out the possibility of experimental error related with changes in the batches of ticks used for the artificial infestations. This meant that not all the individual regressions were significant, but the

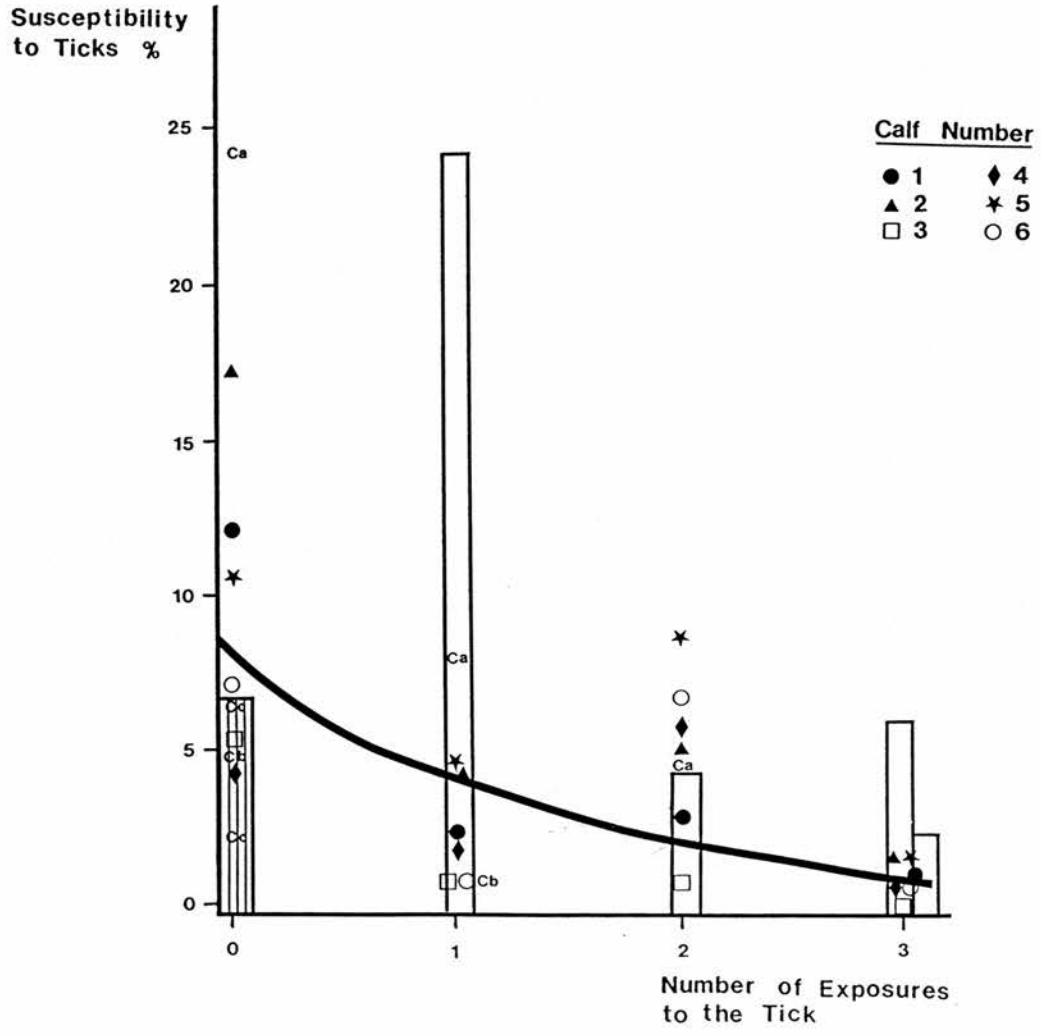


Figure 7.3. Curvilinear regression to fit the values of susceptibility to the tick Boophilus microplus in calves repeatedly exposed to larvae, and subjected to skin tests with tick-derived antigens. Susceptibility was expressed as a percentage of the ticks surviving to maturity, and was transformed to the power 0.25 for the analysis and calculation of the linear equation, but the graph is presented back in the untransformed parameter. Each symbol represents the values obtained for each of the calves on each test. An individual regression was calculated for each animal (observed values for each calf are represented by different symbols) and the combined regression was highly significant ($P < 0.01$). No differences were detected between the slopes or between the constants of these regressions. The clear bars indicate the values obtained in naive control calves used in each infestation, and Ca, Cb and Cc indicate the values obtained for these calves when exposed subsequently to the tick. The shaded bar indicates the value observed in a control calf repeatedly skin tested.

lack of significant difference in the slopes suggests that this is an uncontrolled experimental variation. Figure 7.3 shows a range of values of susceptibility obtained on animals naive to the tick and a definite decrease in values after repeated exposure. Similarly, the value of susceptibility obtained in the control of repeated skin test was compared with the mean of the naive animals using a modified 't' test (Sokal and Rohlf, 1981), and no significant differences were found between them. This indicates that repeated skin testing had no effect on the susceptibility of the animals to the tick.

7.3.2.- Evolution of the skin reactivity to the injected antigens after repeated exposure of the calves to the tick.

The strength and the dynamics of the reactions elicited by intradermal inoculation of the different antigens varied according to the previous exposure of the animals to the ticks (Figures 7.4 and 7.5). Results are illustrated in figure 7.4 for the parameter diameter of reaction and in figure 7.5 for the parameter compiled reaction.

Responses peaked at 30 minutes post injection in all the skin tests performed. In naive animals, reactions were faint but still able to be demarcated and the two way analysis of variance detected significant ($P < 0.05$) differences between the antigens, between the times of reading of the reaction and their interaction, for both parameters. On animals exposed once to the tick, after the peak at 30 minutes, reactions were reduced to a level similar to that reached at 24 HPI by animals exposed twice and three times to

Figure 7.4.- Skin reactivity to different antigens derived from the tick Boophilus microplus when tested sequentially on calves with increasing exposure to tick larvae. Values shown are the means of the diameters of the areas of oedema and erythema which developed on the calves. PK1, PK2 and PK3 identify antigens purified from larval extracts by anion exchange chromatography. GSG denotes an homogenate of salivary glands from female ticks and PBS the saline control. In each category of exposure to ticks, highly significant differences ($P < 0.01$) were observed between the antigens and between the size of the reaction at various times after injection.

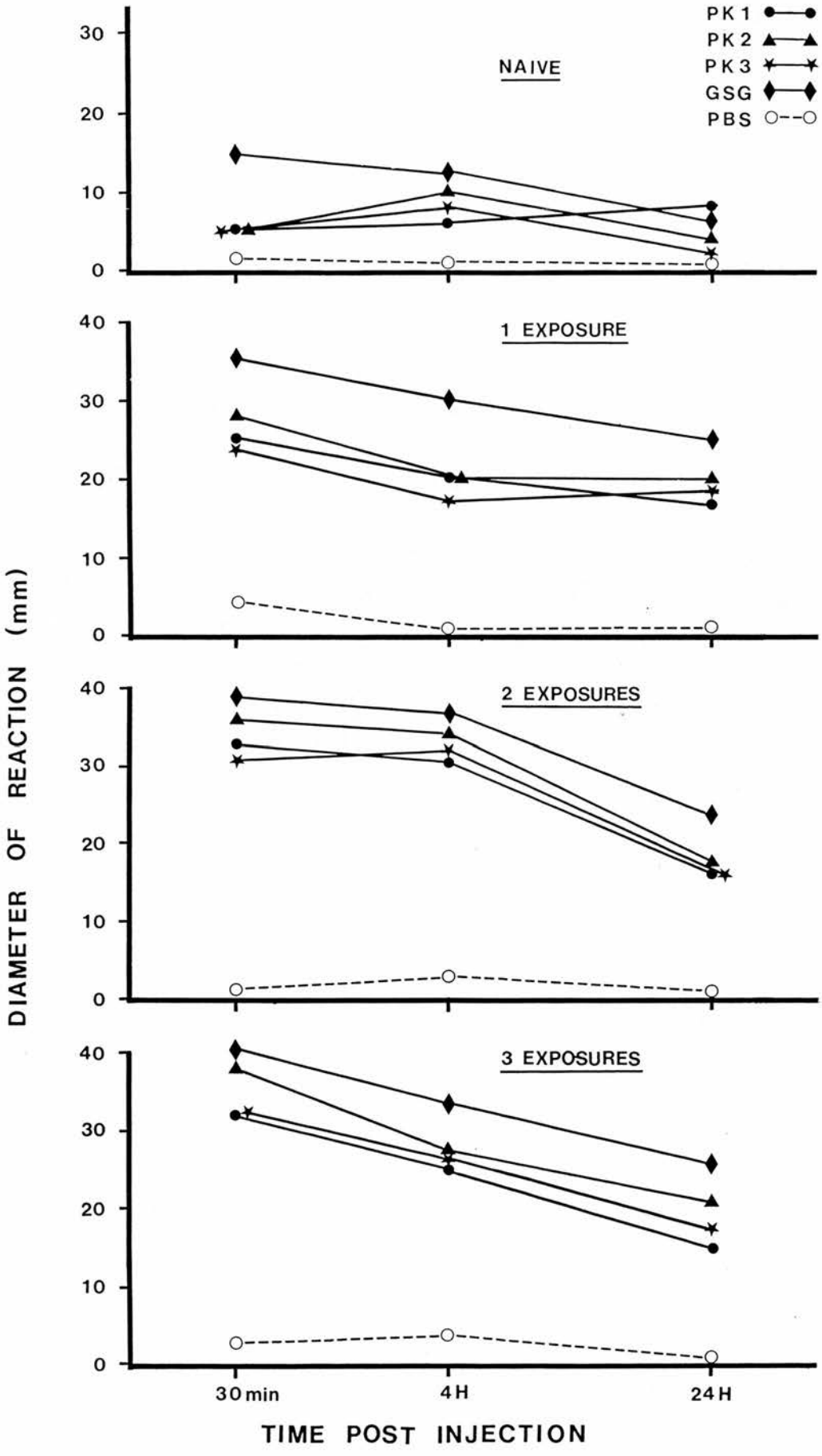
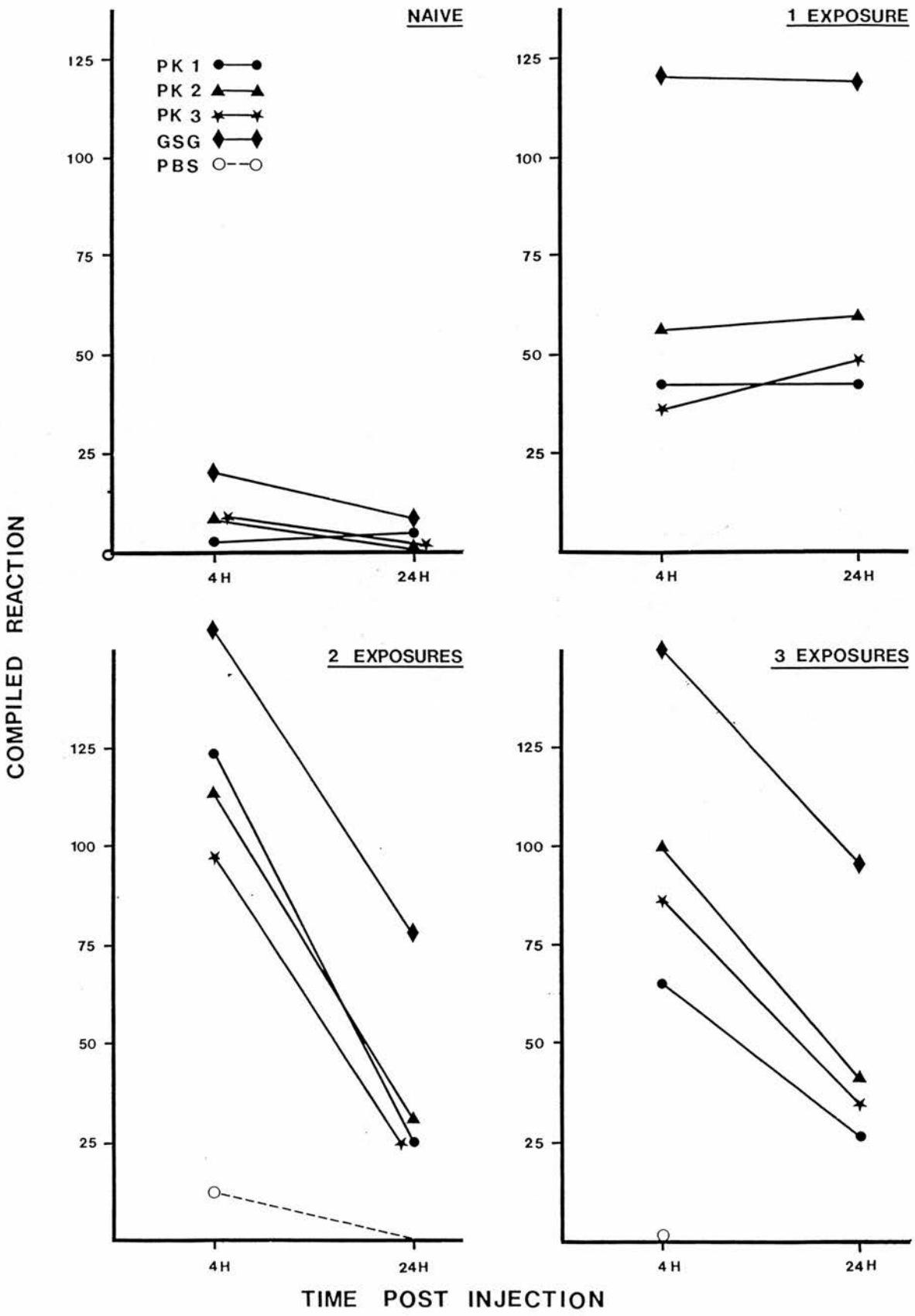

PK 1
PK 2
PK 3
GSG
PBS
NAIVE
1 EXPOSURE
2 EXPOSURES
3 EXPOSURES
DIAMETER OF REACTION (mm)
0
10
20
30
40
30 min
4H
24H
TIME POST INJECTION

Figure 7.5. Skin reactions to tick-derived antigens on calves subjected to repeated exposure to Boophilus microplus larvae and to repeated skin tests. The means of the parameter compiled reaction are displayed. PK1, PK2 and PK3 = larval antigens purified by salt chromatography. GSG = salivary gland homogenate. PBS = saline control. The two way analysis of variance showed highly significant differences ($p < 0.01$) between antigens and between both times of reading of the reactions in all the categories of previous exposure to the tick but when the animals had been exposed once to the tick, no differences on both readings were detected.


NAIVE
1 EXPOSURE
2 EXPOSURES
3 EXPOSURES
PK 1
PK 2
PK 3
GSG
PBS
125
100
75
50
25
4H
24H
COMPILED REACTION
TIME POST INJECTION

the tick. At this time, significant differences were detected between antigens but not between times of measuring the compiled reaction. The differences in the levels of reaction at the different times of reading increased as the animal's exposure to the tick was increased. Stronger reactions were always found for the antigen GSG.

The diameter of the dermal reactions elicited by each allergen at 30 minutes post injection was used to calculate a regression line on each individual for the evolution of the reactions in relation to the sequential exposure to the tick. A combined regression was fitted for each antigen using data from all the calves of the main group and from the control for the second infestation (CA). It was shown to be highly significant for all the antigens and demonstrated no differences between the slopes or the constants of the regressions of individual calves.

The regressions to different antigens were then compared, and these results are illustrated on figure 7.6. There was an increase in the size of the reaction related with the increased exposure to the tick, and a significantly higher level of reactivity to GSG on animals unexposed to the tick. The dermal responses in the calf repeatedly skin tested were also analysed and showed no increase on the reactivity after repeated testing. The reactions displayed by this calf in the final test were compared with the mean obtained by animals exposed twice to the tick (that were subjected to the same number of skin tests), using the modified 't' test (Sokal and Rohlf, 1981). For all antigens, responses were significantly lower in the control calf ($P < 0.05$).

7.3.3.- Development of anti-tick antibodies on the calves

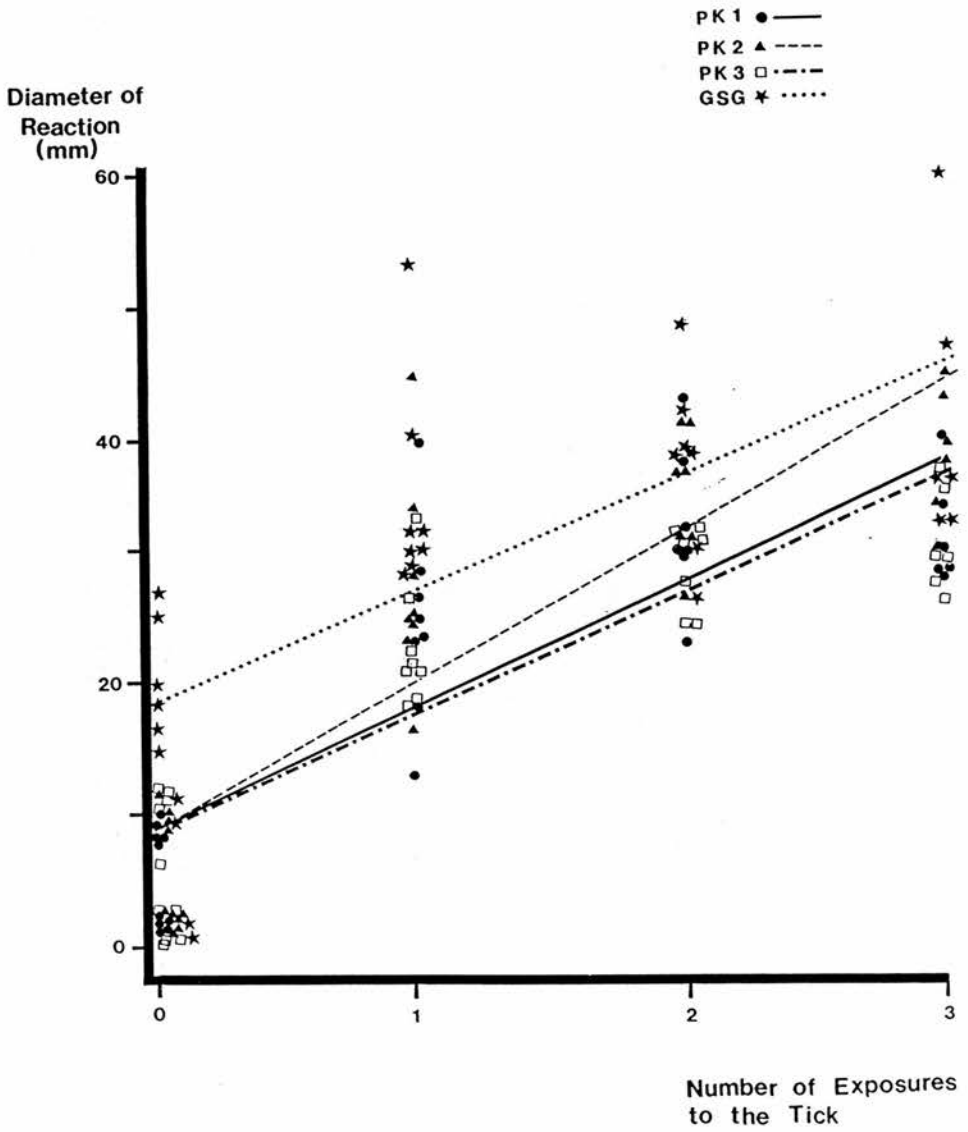


Figure 7.6. Comparison of the dermal reactions elicited at 30 minutes after the injection of antigens derived from Boophilus microplus on calves sequentially exposed to the tick. Each line represents the regression calculated for each antigen using 7 animals (6 of main group and CA. See text). PK1, PK2 and PK3 are antigens purified from larval extracts by anion exchange chromatography. GSG is a salivary gland homogenate from female ticks. The analysis of variance of the deviates from the lines showed that all the responses could be predicted by a common regression ($P < 0.01$). The regressions to the different antigens showed the same slope, but they differed ($P < 0.01$) on the level of the constants.

repeatedly skin tested and exposed to the tick.

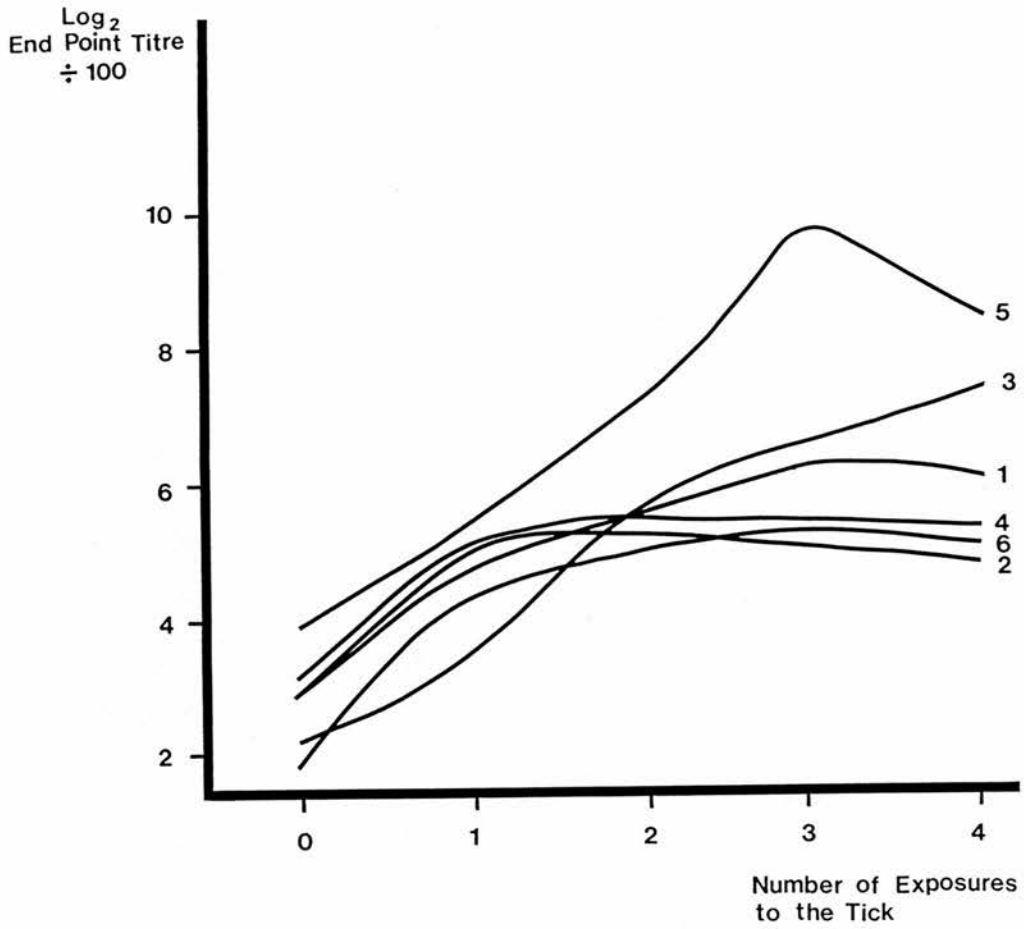
Results obtained in the ELISA test are presented in figure 7.7, and are expressed as endpoint titre values. All animals showed an increase in the antibody content after they were exposed to ticks, but two different pattern of responses were observed. Calves 2, 4, 6 and A (whose individual linear regressions of the responses were not significant), increased their antibody titre after the first exposure to the tick, but tended to remain at the same level after subsequent infestations. Calves 1, 3, 5 and particularly B, showed increasing antibody titres. The analysis of the combined regression for all the animals showed highly significant differences ($P < 0.01$) in the slopes of individual regressions.

The control calf subjected to three repeated skin tests and only exposed to ticks on the final one, showed a similar pattern of antibody development to the above mentioned animals but at a significantly lower level ($P < 0.05$). The antibody level attained for this calf after the third test, is equal to those observed in the control calves exposed only once to the tick.

7.3.4.- Study of the relationship between the acquired resistance to the tick, the dermal responses in the skin test and the level of anti-tick antibodies in the animals.

A multiple matrix of correlations was prepared in the same way as described previously (5.2.3) and these results are summarized in table 7.2. Correlations were calculated on two forms: using only data from the final test for the eleven experimental animals (grouping one), or gathering data from the four tests ignoring the

Figure 7.7. Levels of antibodies to salivary gland antigens from the tick Boophilus microplus in calves sequentially skin tested using tick-derived antigens and sequentially exposed to larvae. The top graph illustrates the evolution of the development of antibodies in 6 calves. Each line was manually fitted through the observed values and individual linear regressions of the responses of each animal were significant ($P < 0.05$) for calves 1, 3 and 5. The bottom graph shows the evolution of the responses on individual control calves exposed once (C), twice (B) or three (A) times to the tick. The combined linear regression of the antibody titres in all these animals (10) is illustrated by a solid line in the bottom graph. It was highly significant ($P < 0.01$) but showed highly significant differences in the slopes of antibody development for the various animals. This line was compared with the regression of antibody development in a calf subjected to repeated skin test and only exposed to ticks on the last test (indicated by a broken line on the bottom graph). No differences between the slopes of these two regressions were observed, but they differed ($P < 0.05$) in their constants.


Log 2
End Point Titre
÷ 100
10
8
6
4
2
0
1
2
3
4
5
3
1
4
6
2
Number of Exposures
to the Tick

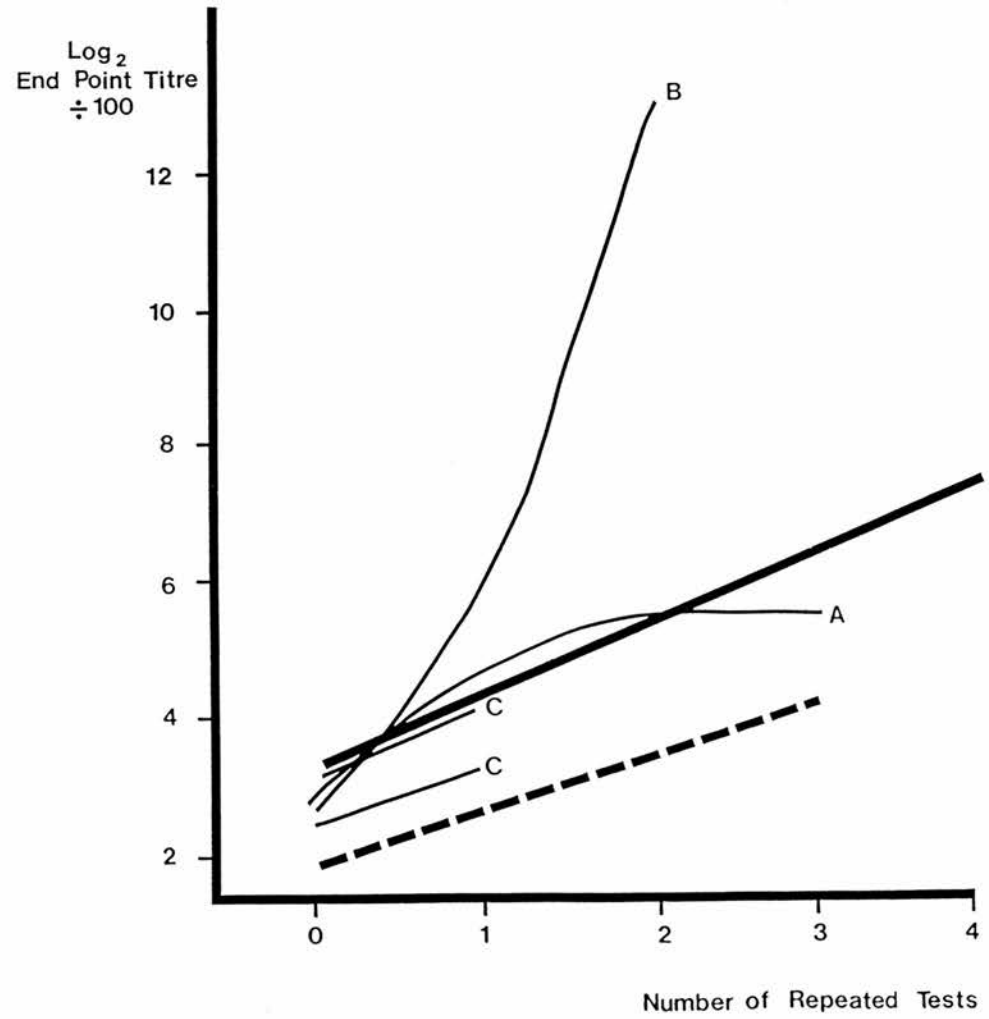

Log 2
End Point Titre
÷ 100
12
10
8
6
4
2
0
1
2
3
4
B
A
C
C
Number of Repeated Tests

Table 7.2.- Relationship between the susceptibility to the tick Boophilus microplus and the size of reactions elicited after the intradermal inoculation of antigens derived from the tick, in calves repeatedly exposed to larvae.

ANTIGEN	Correlation coefficient of Transformed susceptibility -------- vs. --------- Diameter at 30 minutes	Compiled reaction at 4 hours	Grouping @
PK1	-0.71*	-0.74**	1
	-0.54**	-0.23	2
PK2	-0.61*	-0.80**	1
	-0.59***	-0.43*	2
PK3	-0.64*	-0.75**	1
	-0.62***	-0.43*	2
GSG	-0.74**	-0.65*	1
	-0.66***	-0.55**	2

@ : 1 = Data of 11 animals at final test.
2 = 32 data of animals repeatedly sampled (6+7+8+11)

* = (P < 0.05)
** = (P < 0.01)
*** = (P < 0.001)

fact that some data were drawn repeatedly from the same animals (grouping two, total data = 32).

Significant relationships were found between the susceptibility to the tick (transformed parameter) and the reactivity on the skin test by measuring either the diameter of the reaction at 30 minutes or the compiled reaction at 4 HPI for all the antigens used. When data from all the tests were correlated, the significance increased only for reactions at 30 minutes.

Antibody titres observed in the ELISA test were also correlated with the susceptibility to the tick and the size of the skin responses to the various antigens. The titres were correlated in two ways: The first, corresponded to the titres observed using sera obtained the day before the skin test and the application of larvae were conducted. This was named antibody titre A. This titre represents the effect of the level of existing antibodies on the susceptibility to the tick or on the size of the reactions. The second, (antibody titre B), was the level of antibodies developed as a consequence of the skin test and/or the tick infestation, corresponding to the values obtained on the sera sampled four weeks after the skin test and the application of larvae were conducted.

In this way, using as example data from the final test (grouping one), antibody titre A were those titres observed before the final skin test on: 6 animals with three previous exposures to the tick, one calf with two and one calf with one previous exposures, and two calves naive to the tick. At the same time antibody titre B, were those titres observed on the same animals four weeks after the challenge tick infestation and the skin tests were conducted.

A significant negative correlation between the susceptibility to the tick and the antibody titre A was found when data from all tests were included ($r = -0.49$, $P < 0.01$), but this relationship was not significant using only data from the final test ($r = -0.50$, $P > 0.10$). Correlations with the titre of antibodies developed after exposure to the tick were not significant (respectively; $r = -0.31$, $P < 0.10$, > 0.05 and $r = -0.46$, $P > 0.10$).

The correlation coefficients of the relationship between antibody titres and the size of the dermal reactions to different antigens are presented in table 7.3. When only data from the final test were used for the analysis, significant coefficients ($P < 0.05$) were demonstrated between the antibody titre A, and the size of the reactions at 30 minutes, for the larval derived antigens PK1, PK2 and PK3, and with the compiled reaction at 4 HPI for PK3 and GSG. However, when data from all the tests were included in the analysis, highly significant correlations ($P < 0.01$) were observed between the antibody titre A and the reactions to all antigens both at 30 minutes and 4 HPI and between the antibody titre B and the reactions at 30 minutes to all antigens.

The multiple matrix of correlations obtained allowed the observation of a more complex pattern of relationships between the parameters analysed here. A significant relationship was found between the normal skin fold thickness of each animal (six values per calf) and the susceptibility to the tick ($r = -0.31$, $P < 0.05$, using data from the final test only). The normal thickness of the skin on the calves also showed increases apparently related to the repeated exposure to the tick and to the repeated skin testing

Table 7.3.- Relationship between the size of the reactions elicited by the intradermal inoculation of various antigens derived from Boophilus microplus ticks and the antibody level to salivary gland antigens found in the sera of calves: before the performance of the test (Antibody titre A) or four weeks after the test and the exposure of the animals to tick larvae (Antibody titre B).

Reaction	ANTIGEN	Grouping 1@(n=11)		Grouping 2@(n=32)	
		Antibody titre A	Antibody titre B	Antibody titre A	Antibody titre B
Diameter at 30 minutes	PK1	0.74**	0.63*	0.75***	0.52**
	PK2	0.85***	0.67*	0.84***	0.57***
	PK3	0.69*	0.52	0.80***	0.52**
	GSG	0.59	0.68*	0.68***	0.54**
Compiled reaction 4 hours	PK1	0.56	0.36	0.48**	0.30
	PK2	0.56	0.47	0.66***	0.50**
	PK3	0.69*	0.32	0.68***	0.39*
	GSG	0.70*	0.51	0.72***	0.46**

@ : Grouping 1 = data of 11 animals at final test
Grouping 2 = 32 data of animals repeatedly sampled

* = (P < 0.05)
** = (P < 0.01)
*** = (P < 0 001)

(figure 7.8).

Finally, data were examined in the search for a predictor of the level of resistance attained for each animal at the final infestation. To accomplish this, the susceptibility to the tick observed on the last test for each of the calves of the main group, were correlated with the size of the skin test responses and the antibody levels observed in the animals from the first test when the animals had had no previous exposure to the tick.

Although a small number of animals were studied, three associations with the level of susceptibility to the tick attained after repeated exposure became evident: Negative correlations with the diameter of the reaction to GSG ($r = -0.83$, $P < 0.05$), and to PK2 ($r = -0.70$, $P < 0.10$) at 30 minutes post inoculation, and a positive correlation with the level of anti-tick antibodies developed for the calves after the initial exposure to the tick. (antibody titre B) ($r = 0.75$, $P < 0.10$).

7.4.- DISCUSSION

The acquisition of resistance to the tick in the calves during this experiment followed a pattern similar to those described elsewhere (Hewetson and Nolan, 1968; Hewetson, 1971; Wagland, 1975). The values of susceptibility observed in the naive calves (range: 1.92 - 24.4%) agree with those reported at the first infestation by Hewetson and Nolan (1968) using crossbreed bulls (range: 2.0 - 21.0%). However, the mean value (calculated using the exponentially transformed data and presented in the natural scale), is well below those reported, which appears to be due to the method of analysing

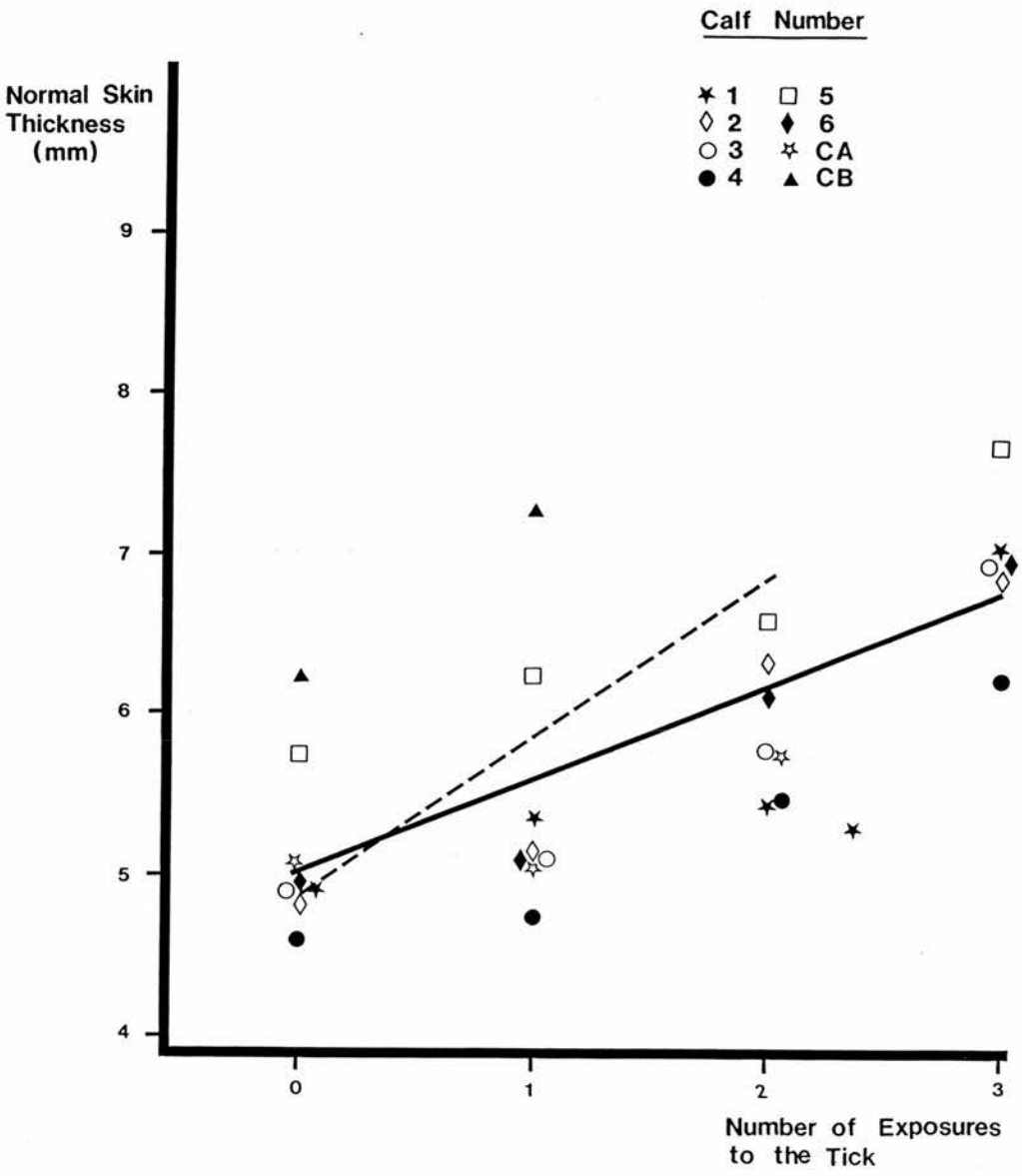


Figure 7.8. Linear regression to predict the normal skin fold thickness of calves repeatedly exposed to Boophilus microplus ticks and repeatedly inoculated intradermally with tick-derived antigens. A regression line was fitted for each individual (indicated as different symbols) and a combined regression for all animals (indicated as a solid line) was highly significant ($P < 0.01$), displaying highly significant differences in the constant values for the different animals. The regression for a control calf subjected only to repeated skin tests (indicated as a broken line) was not different from the combined regression.

the data.

After the fourth infestation the susceptibility values were reduced to a mean of 0.89% (range: 0.22 - 1.5%), which lies on the range of values expected for resistant Bos indicus x Bos taurus crossbreed cattle (Utech, Wharton and Kerr, 1978). The values of susceptibility observed in the experimental animals were lower than those observed on the Holstein calves during the previous experiment, but similar to those observed on the Criollo crossbred cattle (6.3.2).

Reactions induced by the inoculation of tick-derived antigens followed a pattern similar to that described for the Holstein calves in the previous chapter. The use of the diameter instead of the area as a measure of the reactions facilitated the statistical analysis of the data for the production of more homogeneous variances between the groups compared and also for the normalization of the distribution of data.

Previous works on skin tests related with the immune status of the animals to the ticks have used different parameters to measure the reactions, mainly the diameter on the area of the reactions (Willadsen and Williams, 1976; Willadsen et al., 1978) or the changes in the thickness of the skin (Binta and Cunningham, 1984; Wikel, Graham and Allen, 1978). During this experiment results did not provide evidence to support the use of a particular measure since significant correlations with the susceptibility of the animals to the tick were found in both parameters used; the diameter of the reaction at 30 minutes and the compiled parameter (diameter x thickness) at 4 HPI.

It is clear that different types of responses are measured at

30 minutes and at 4 HPI. Immediate hypersensitivity responses peaking at 30 minutes were observed in all antigens used, but an underlying reaction was still detected at 24 HPI. It was demonstrated during the previous chapter (6.3.2) that the compiled parameter was an adequate measure of the reaction at 4 HPI when attempting the correlation with the susceptibility to the tick.

The use of the compiled parameter displayed wider differences between the responses at 4 and at 24 HPI in animals exposed to the tick (figure 7.5) but at the same time variances within groups were increased. This appeared to be caused by the imprecision and subjectivity of the delineation of the dermal response when attempting to measure the area or the diameter of the reaction. Any initial immediate hypersensitivity response can be easily delineated and measured, but when this reaction starts to be infiltrated by inflammatory cells, related with Arthus or delayed type hypersensitivity responses (Roitt, Brostoff and Male, 1985), the reactions tend to be poorly delimited.

The reactions displayed for the Gyr-Holstein calves when naive to the tick were fainter than those seen in the Holstein calves during the first evaluation described in chapter six, using the same antigens. After the first exposure to the ticks in this experiment, the size of the dermal reactions rose to levels similar to those seen in the group of Criollo cattle adapted to tropical conditions, and in subsequent tests, the size of the reactions increased steadily after each exposure to the tick indicating acquisition of immunity. Stronger reactions were always observed to GSG and, for all antigens, a clear separation between the responses

recorded in animals when naive or when immune to the tick, was noted.

Antibody titres to the salivary gland antigen in the sera of the calves followed the same evolution as the skin test reactions. The control calf repeatedly subjected to intradermal inoculations of tick-derived antigens also showed an increase in titre as a consequence of the repeated tests but only approaching a level seen on animals with one exposure to the tick. These findings of antibody evolution are in concordance with those of Brossard (1976), who used an indirect immunofluorescence assay and detected the appearance of specific anti-salivary gland antibodies after the first exposure of cattle to B. microplus.

During this experiment, the size of the reactions elicited by all antigens used (PK1, PK2, PK3 and GSG) as measured both at 30 minutes or at 4 HPI, showed correlation with the susceptibility to the tick (transformed parameter). In the previous experiment using the same antigens (6.3.2), the susceptibility to the tick was only correlated with the size of reactions to PK1, PK3 and GSG in the first trial, but these results were not reproduced later.

The higher range of correlations found in the present experiment were expected because the group of Gyr-Holstein calves were able to demonstrate higher levels of resistance than those displayed by the Holstein calves in the previous chapter, and because they were kept on a high nutritional plane (as already discussed in chapter six). However, the small number of animals used in the experiment limits extrapolation of results to any field situation.

The form in which the data is presented for the correlation

analysis merits some consideration. Correlations already discussed correspond to those calculated using data from 11 animals at the final stage of the experiment (6 calves of the main group with 3 previous exposures to the tick and the same number of skin tests, the CA control with two exposures and skin tests, the CB control exposed once and subjected to one skin test, the ST control on which two skin tests had been performed and two naive animals). Alternatively, correlation coefficients were also calculated using a total of 32 paired data, corresponding to the values displayed for the animals in the different stages of the experiment as they were exposed to ticks and acquired resistance. Higher levels of significance of the correlations were found when the second grouping of the data was used. This could have been caused by the higher degrees of freedom and the consequent lower levels for significance implied. However, the paired data from the different stages on the acquisition of resistance by the experimental animals are likely to be correlated as those in the final test, with the exception of the animals naive to the tick where non specific skin reactions could be elicited by pharmacologically active agents contained in tick derived antigens (Ribeiro, 1987).

None of the above mentioned grouping of the data are likely to represent the situation faced when evaluating cattle for resistance to ticks in a tick endemic area. Another approach that could be advocated to study this relationship would be the plot of the slopes showed for each animal on each parameter after the repeated exposure to the tick, but again this would not be the situation when facing a selection task in field situations.

Antibody titres to the salivary gland antigen present in the sera of the animals on the day of the test were correlated negatively with the susceptibility to the tick and positively with the observed size of the skin reactions. This could indicate the involvement of serum antibodies, particularly immunoglobulin G (IgG), both in the resistance to the tick and in the development of the hypersensitivity reaction to tick-derived antigens. However, in the context of this experiment, this repeated stimulus (repeated exposure to ticks) would produce an increase in all three parameters, without indicating that the level of one could be predicted by the level of the other if measured after the challenging stimulus has dissapeared (when all parameters have reached a maximum and stable level on each animal), that is the situation found on animals adapted to tropical conditions. These could be the reasons for the disagreement between these results and those of Willadsen et al. (1978), who found no correlation between the concentration of antibody to a purified tick antigen measured by indirect haemagglutination and the resistance of the animals to B. microplus ticks.

The negative correlation found between the susceptibility to the tick and the normal skin thickness of each animal (the skin fold thickness before the injection of the inocula) may be subjected to a similar phenomenon as that described above. The thickness of the normal skin increased as the animals were repeatedly exposed to the tick.

Although there are reports in the literature about the histology of the early changes in the skin associated with tick infestation on naive and resistant cattle (Amin-Babjee and Riek,

1986; Schleger et al., 1976), there are no reports specifically on this long term effect of tick infestation on the skin, but Bonsma (1981) states that thickness and structure of the hide are important factors when attempting the selection and breeding of tick-repellent cattle. On the other hand, the observed increase in skin thickness could be a natural event in animals actively growing.

A clear relationship was observed between the size of the reactions to PK2 and GSG, recorded when the animals had not yet been exposed to the tick, and the level of resistance (decrease in susceptibility) attained for the animals after three repeated exposures to the tick. This result is encouraging and merits further research.

Could the skin tests be used as predictors of the levels of resistance that the animals are going to acquire in the future? Skin reactions found in the animals when naive to the tick may indicate the individual sensitivity to pharmacological compounds or to inflammatory mediators contained in the extracts, and they are not immune mediated. It is possible that this individual sensitivity will affect the future feeding performance of the tick on the animal. However, these results are limited by the small numbers of animals observed.

7.5.- CONCLUSIONS

- Crossbred Gyr-Holstein calves acquired resistance after repeated exposure to B. microplus ticks, that was expressed as a decrease in the percentages of larvae surviving to maturity. At the same time, the calves developed immediate

hypersensitivity reactions to the intradermal injection of tick derived antigens. The size of reactions increased after each exposure. Data did not provide evidence to support the use of a particular parameter to measure the reactions.

- Antibody titres to GSG measured by ELISA increased in association with the repeated exposure of the animals to the tick with different patterns observed between individuals. A control calf repeatedly skin tested also showed increase in its antibody titres, but values obtained after three tests were similar to those in calves exposed only once to the tick.
- Significant correlations between susceptibility to the tick and the size of the reaction obtained in the skin test were found for all the antigens used (PK1, PK2, PK3 and GSG) in both parameters recorded. The titres of serum antibodies to GSG were correlated negatively with the susceptibility to the tick and positively with the size of the reactions.
- It was concluded that in the context of this experiment data from skin tests, the titre of serum antibodies to GSG and the susceptibility to the tick were not independent, since they were all related to a common factor; the sequential exposure to ticks of the experimental animals. In attention to this, further experiments to evaluate the value of skin tests for the selection of animals on the basis of their capacity to acquire resistance to the tick, should be performed on animals in which the immunity to the parasite had been developed and had reached a stable level.
- Finally, the relationship between the dermal responses to

PK2 and GSG in the animals when naive to the tick, and the level of susceptibility to the tick reached after repeated exposures was highlighted as a potential predictor of the level of resistance to be acquired by an individual. However, further research in this topic is required.

CHAPTER EIGHT:

EVALUATION OF THE SKIN TEST REACTIVITY OF DIFFERENT PROTEINACEOUS MATERIALS PURIFIED BY CHROMATOGRAPHY ON DEAE-SEPHADEX FROM LARVAE OF THE TICK Boophilus microplus.

SUMMARY

The relationship between susceptibility to Boophilus microplus and the dermal reactivity to antigens isolated from larval extracts of the tick by anion-exchange chromatography, was studied using two different experiments with Holstein and crossbred cattle previously exposed to the tick and grazed under tropical conditions. When injected intradermally all the antigens elicited immediate hypersensitivity reactions. Different levels of susceptibility to the tick were observed between the groups of cattle, but there were inaccuracies in the assessments, associated with various managerial factors. Parametric and non-parametric statistics were used to study the relationship of skin reactivity with the susceptibility to the tick of the animals, and it was concluded that the antigen P3 was a candidate antigen to be used in the following experiments to study that relationship.

8.1.- INTRODUCTION

In chapter four, the fractionation of larval extracts from the tick Boophilus microplus using anion-exchange chromatography was described. In that experiment, four different fractions were obtained using DE-52 anion-exchange chromatography. However, up to seven fractions were produced when DEAE-Sephadex was used as the

anion-exchanger.

The preliminary evaluation of the reactivity in animals exposed to the tick, of the antigens isolated at CTVM and the relationship of the size of the elicited responses with the level of resistance to the tick of the animals was described in chapter six. There, all the fractions examined showed the capacity to elicit immediate hypersensitivity responses in the skin of animals exposed to the tick and the size of the reactions produced by the antigens PK1 and PK3 (eluted from the columns at molarities of sodium chloride of respectively 50 mM and 150 mM) showed correlation with the level of resistance to the tick.

This chapter describes the evaluation of materials isolated at LIMV for their capacity to induce dermal reactions in animals with previous exposure to the tick. Fractions obtained from chromatographies of larval extracts L2-65 and L4-65 (4.3.2.2) were studied.

Evaluations were conducted at the ICA's research centre "La Libertad" in Villavicencio, where B. microplus ticks are endemic and reproduce on the pastures throughout the year.

8.2.- MATERIALS AND METHODS.

8.2.1.- Design of the experiments.

The evaluation of materials obtained after chromatography of the larval extracts either L2-65 or L4-65 (4.3.2.2) were considered as separate experiments. In each, the candidate antigens were skin tested on the animals for their capacity to induce hypersensitivity

reactions, and data on susceptibility to the tick of each individual were collected using both the artificial infestation method and the evaluation of tick burdens resulting from field infestations (8.2.4).

Two types of animals were tested in each experiment; Holstein and crossbred (8.2.2) which were grazed on one of three paddocks of Brachiaria decumbens adjacent to the corral (8.2.2). The skin tests and the tick counts were performed as close in time as permited by managerial problems of tick burdens on the pastures. The final chronology of the experiments is presented in table 8.1.

Experiment one consisted of the evaluation of materials resulting from the chromatography L2-65. Skin tests were conducted during September 1987. A total of 28 animals split into three groups were used; 16 Holstein and 10 crossbred cattle previously exposed to ticks (8.2.2) and two Holstein calves naive to ticks and not subsequently exposed were used as controls for the non specific reactivity of the materials. The tick exposed animals were subject to an evaluation of tick burdens resulting from field infestation during July 1987 for the Holstein group and September 1987 for the crossbred group. Susceptibility to ticks using the artificial infestation method was measured in the Holstein group in May, and in the crossbred group in October 1987.

Experiment two consisted on the evaluation of materials from column fractionation L4-65. This time 31 animals were evaluated, 15 Holstein and 16 crossbred, all previously exposed to ticks (8.2.2). Skin tests were conducted in the crossbred between October and November 1987, and in the Holsteins in December 1987. Counts of tick field burdens were performed on the crossbred group in October

Table 8.1. Chronology of the activities related with the evaluation of the allergenic capacity of proteinaceous materials separated by anion-exchange chromatography from the Boophilus microplus larval extracts L2-65 or L4-65.

			ACTIVITIES PERFORMED ON EACH GROUP OF ANIMALS *		
Year	Month	Week	Holstein	Crossbred 1	Crossbred 2
1987	July	19-25	FC (1)		
		26- 1	FC (1) A.		
	Aug.				
	Sept.	30- 5	ST (1)	ST (1)	
		6-12	ST (1) A.	ST (1)	
		13-19		FC (1)	
		20-26		FC (1) A.	
	Oct.	27- 3	ST (1) A.	ST (1)	
		4-10			
		11-17		AI (1)	
		18-24			
		25-31			ST (2)
	Nov.	1- 7		AIC (1)	FC (2)
		8-14	FC (2)		FC (2) A.
		15-21	FC (2) B.		
		22-28			
	Dec.	29- 5			AI (2)
		6-12	ST (2)		
		13-19			
		20-26			AIC (2)
1988	Jan.	27- 2			
		3- 9			
		10-16			
		17-23	AI (2)		
		24-30			
	Feb.	31- 6			
		7-13	AIC (2)		

*: FC = Counts of tick field burdens.
ST = Skin test performance.
AI = Artificial infestation with 10,000 larvae.
AIC = Count of ticks from AI.
A. = Animals sprayed with coumaphos.
B. = Animals sprayed with deltametrine.
Numbers in brackets indicate the experiment to which the activity is related

1987, and in the Holstein group in December 1987. The artificial infestation was conducted in the crossbred group in December 1987 and in the Holstein in January 1987.

8.2.2.- Experimental animals and pastures.

The Holstein group of animals used in experiments one and two, were the same group of calves described in chapter six (6.2.2), which were not restricted for experimental use and were grazed all the time on the experimental pastures. They were two years old by the time of evaluation. Two male one year old Holstein calves which were not exposed to ticks and maintained at LIMV in Bogotá were used as controls in experiment one.

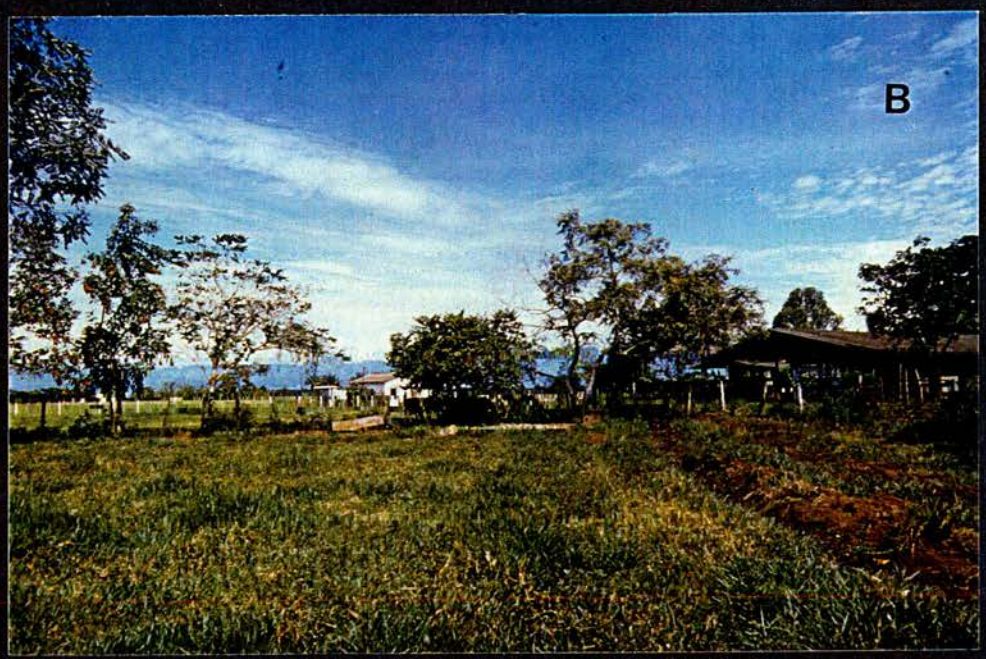
Two groups of male crossbred animals belonging to a dual purpose herd were used, but they were only available for experimental evaluations for short periods. One group was used in experiment one and the second in experiment two. Animals in both groups were approximately 18 months old and they were a mixture of different breeds (trihybrids and tetrahybrids) including B. indicus and B. taurus and of the latter both European and Criollo types (Figure 8.1).

After arriving at the experimental pastures, animals of the crossbred groups were skin tested and the tick burden carried over by each of them was estimated (8.2.4). They were returned to their original pastures after the completion of the artificial infestation test.

Three different paddocks of Brachiaria decumbens grass, each of 1.5 hectares, were used for the experiment (figure 8.1). Two of them

Figure 8.1. Experimental animals and pastures used at the research station "La Libertad" at Villavicencio to evaluate the skin test reactivity of different tick-derived proteic materials. Plate A shows two crossbred animals used in the experiments. Plate B shows the Holstein group of animals and the array of the paddocks with the corral and laboratory facilities on the background.


A


B

were alternatively used for the maintenance of the experimental animals. The third one was kept with a minimum larval tick burden on the pasture by minimizing the grazing of tick infested animals on it. Mineral salt was the only supplement offered to the animals.

8.2.3.- Skin tests.

Skin tests were performed following the same methods described in chapter six (6.2.3) but reactions were measured as modified in chapter seven (7.2.4). Antigens used were the different fractions collected after chromatography of the respective larval extract. The same nomenclature employed in chapter four (4.3.2.2) was used to identify each semi-purified fraction. Experiment one evaluated fractions produced after chromatography of the larval extract L2-65 and experiment two examined those produced after chromatography of extract L4-65. In each experiment, a salivary gland homogenate (GSG) from B. microplus females and a saline control (PBS) were included (3.3).

No attempt was made to standardize the protein concentration of the materials since the objective of the experiment was to compare the purification of the allergenic activity on each material. On the other hand, the lack of freeze drying facilities make it more difficult to concentrate materials with very low protein content but high allergenic activity. All fractions after chromatography were subjected to similar concentration procedures and frozen (4.3.2.2). GSG was prepared two days before the skin test and frozen. Antigens for the skin test were transported from Bogotá to Villavicencio in an ice cold thermos, and kept like that until the following day when

the test was conducted.

The ammount of protein actually injected into the animals for each fraction was estimated by measuring the protein content (3.2) on a portion of each fraction not transported to Villavicencio. They were expressed as micrograms of protein contained the 0.1 ml used for the injection and were as follows: Experiment one; P1 = 7, P2 = 10, P3 < 5, P4 = 27, P5 < P6 = 180, D2 = 200 and GSG = 35. Experiment two; P1 = 1, P3 = 3, P4 = 19, P5 = 61, P6 = 96, D2 = 41 and GSG = 35.

8.2.4.- Tick counts.

During the weeks following the arrival of each group of animals to the experimental pastures, the tick burden carried over for each animal was estimated by counts of semi-engorged females on the entire animal (Wharton and Utech, 1970) performed four times (experiment one) or three times (experiment two) over a period of two weeks. Then the animals were sprayed with a solution of coumaphos (Asuntol, Bayer Colombia) and allowed to graze in the paddock of low tick infestation levels.

Two weeks were allowed to eliminate any residual activity of the acaricide and then the artificial infestation using 10,000 larvae/animal (Utech, Seifert and Wharton, 1978) was started (3.1.3). Animals were returned to the tick contaminated pastures one week after ticks were applied.

The Holstein group of animals experienced excessive tick burdens resulting from field infestations. This made it impossible to conduct the artificial infestation in experiment one, and data

recorded in May for the same group of animals (6.2.4) were used instead. Also, due to high tick infestations this group of animals was sprayed in November with deltametrine (Bayticol, Bayer Colombia) to afford a longer protective period. They were subjected to the artificial infestation protocol for experiment two in January 1988 (Table 8.1).

Field tick counts obtained on different days were transformed to logarithmic expression ($\log_{10}$) and a mean tick count for each animal was so calculated (Wharton, Utech and Turner, 1970). Values of susceptibility to the tick obtained by the artificial infestation method were exponentially transformed prior to the statistical analysis as described in 5.3.1.

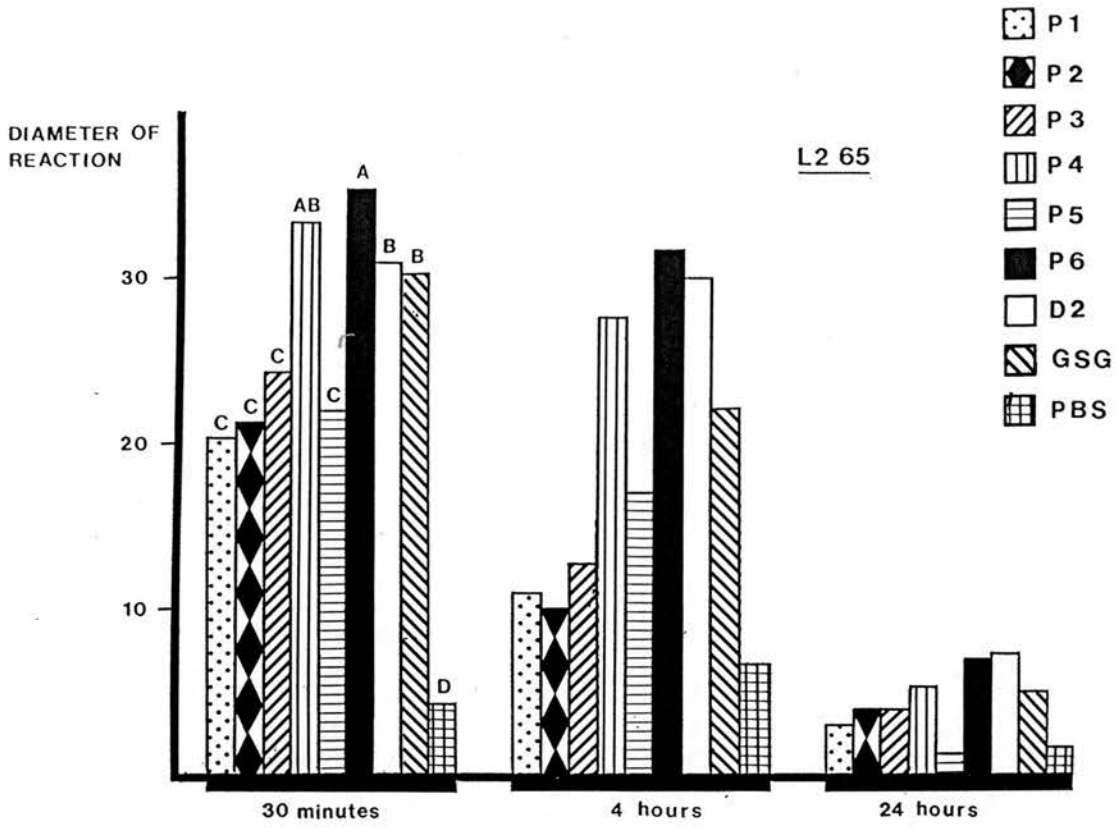
8.3.- RESULTS.

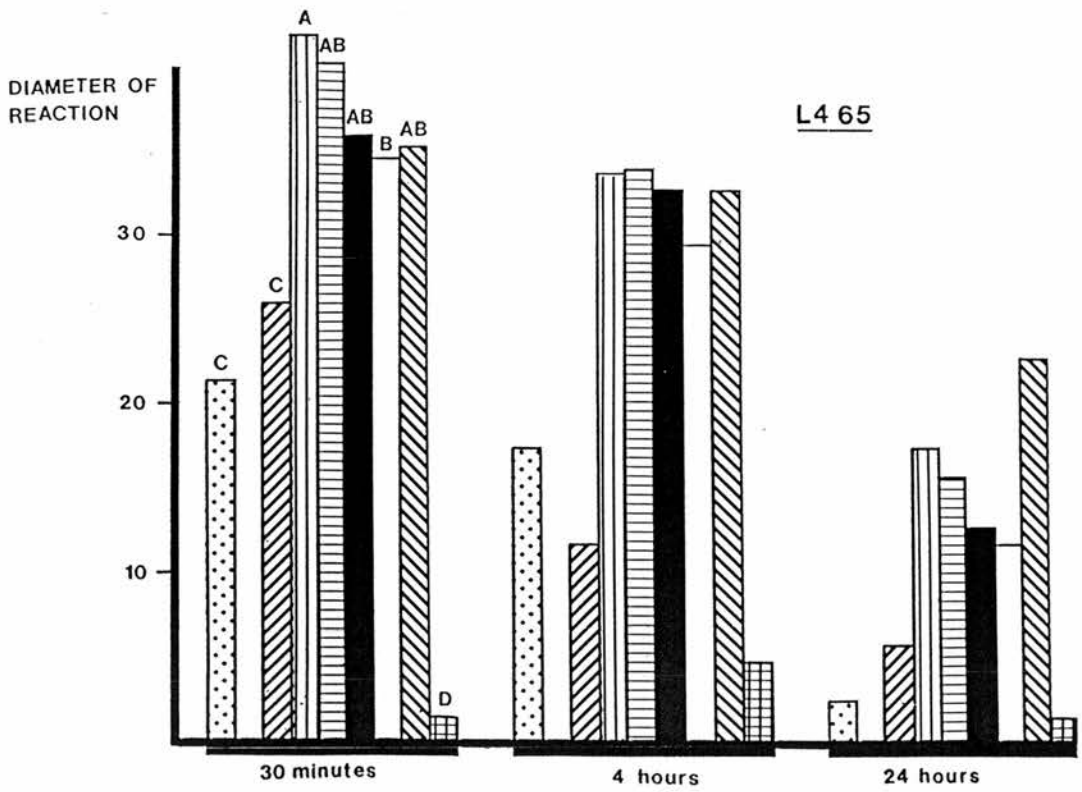
8.3.1.- Skin test responses.

All materials tested, both in experiment one and in experiment two, showed the capacity to induce dermal inflammatory responses in the animals exposed to the tick. Figure 8.2 shows the changes in the size of the reactions observed according to the time after inoculation. In both experiments the reactions peaked at 30 minutes, and at this time the size of the reactions to the antigens were significantly different to those observed at the PBS site of inoculation. Reactions to P1 and P3 were significantly lower than reactions to P4, P6, D2 and GSG.

At 4 HPI the pattern of the reactions was more complex. Variances were higher and were not homogeneous to all antigens. In experiment one, reactions to P1, P2, P3 and P5 could not be

Figure 8.2. Dermal reactions elicited on the skin of cattle exposed to Boophilus microplus ticks, after the inoculation of different mixtures of proteins derived from the tick. The bars illustrate the means on the parameter diameter of reaction, observed in 26 (L2-65) or in 31 (L4-65) animals. Antigens used were materials separated by anion-exchange chromatography of the larval extracts L2-65 or L4-65, (P1, P2, P3, P4, P5, P6 and D2), a salivary gland homogenate from female ticks included for comparative purposes (GSG) and a saline control (PBS). In each set of data a two way analysis of variance indicated highly significant differences ($P < 0.01$) according to both the antigen and the time of measuring the reaction. Means at 30 minutes were compared using the Duncan's multiple range test. In each set of data (L2-65 or L4-65) bars bearing the same letter are not significantly different.


DIAMETER OF REACTION
L2 65
P1
P2
P3
P4
P5
P6
D2
GSG
PBS
30
20
10
C
C
C
AB
C
A
B
B
D
30 minutes
4 hours
24 hours


DIAMETER OF REACTION
L4 65
30
20
10
C
C
A
AB
AB
B
AB
D
30 minutes
4 hours
24 hours

separated from reactions to PBS ($P > 0.05$). In experiment two, reactions to P1 and P3 were not different from reactions to PBS ($P > 0.05$).

Antigens used in experiment one were also tested on two calves naive to the tick. One of these calves showed slight reactions at 30 minutes (diameter under 15 mm) at the sites of injection of antigens P1, P5 and D2. These values will be compared below when examining the relationship with susceptibility to the tick.

The mean of reactions at 30 minutes to the different antigens in both groups of animals (Holstein and crossbred) were compared using a "t" test. Results are presented in table 8.2. In experiment one, significant differences ($P < 0.05$) between both groups of animals were detected only in P6. In experiment two, significant differences between the Holstein and the crossbred groups of animals were observed only in reactions to antigen P1. In general, the Holstein group tended to display stronger reactions.

8.3.2.- Measurement of susceptibility to the tick.

In experiment one, the susceptibility to the tick measured by the artificial infestation method ranged from 1.52% to 10.6%. The Holstein and the crossbred animals showed different distribution of the values (presented on the exponentially transformed scale in figure 8.3B). Variances in both groups were not equal and a modified "t" test demonstrated that the means of both groups differed significantly ($P < 0.001$). The mean susceptibility to the tick was 4.4% on the Holstein group and 6.0% on the crossbred group (statistics of data conducted on the transformed scale, but values

Table 8.2. Mean diameter at 30 minutes post injection of the skin reactions elicited by the inoculation of different proteinaceous materials derived from the tick Boophilus microplus, in cattle exposed to the tick. Results for each material are compared according the group of animals evaluated (Holstein or crossbred).

a) Experiment one (Holstein = 16 crossbred = 10)

Antigen @	Holstein	Crossbred	"t" Statistic	P
P1	20.4	20.6	0.086	0.932
P2	21.7	20.9	0.367	0.717
P3	25.6	22.9	1.185	0.247
P4	32.9	34.6	0.432	0.670
P5	22.0	21.8	0.065	0.948
P6	38.9	30.0	2.622*	0.015
D2	32.9	28.1	1.716	0.099
GSG	32.1	27.9	1.852	0.076

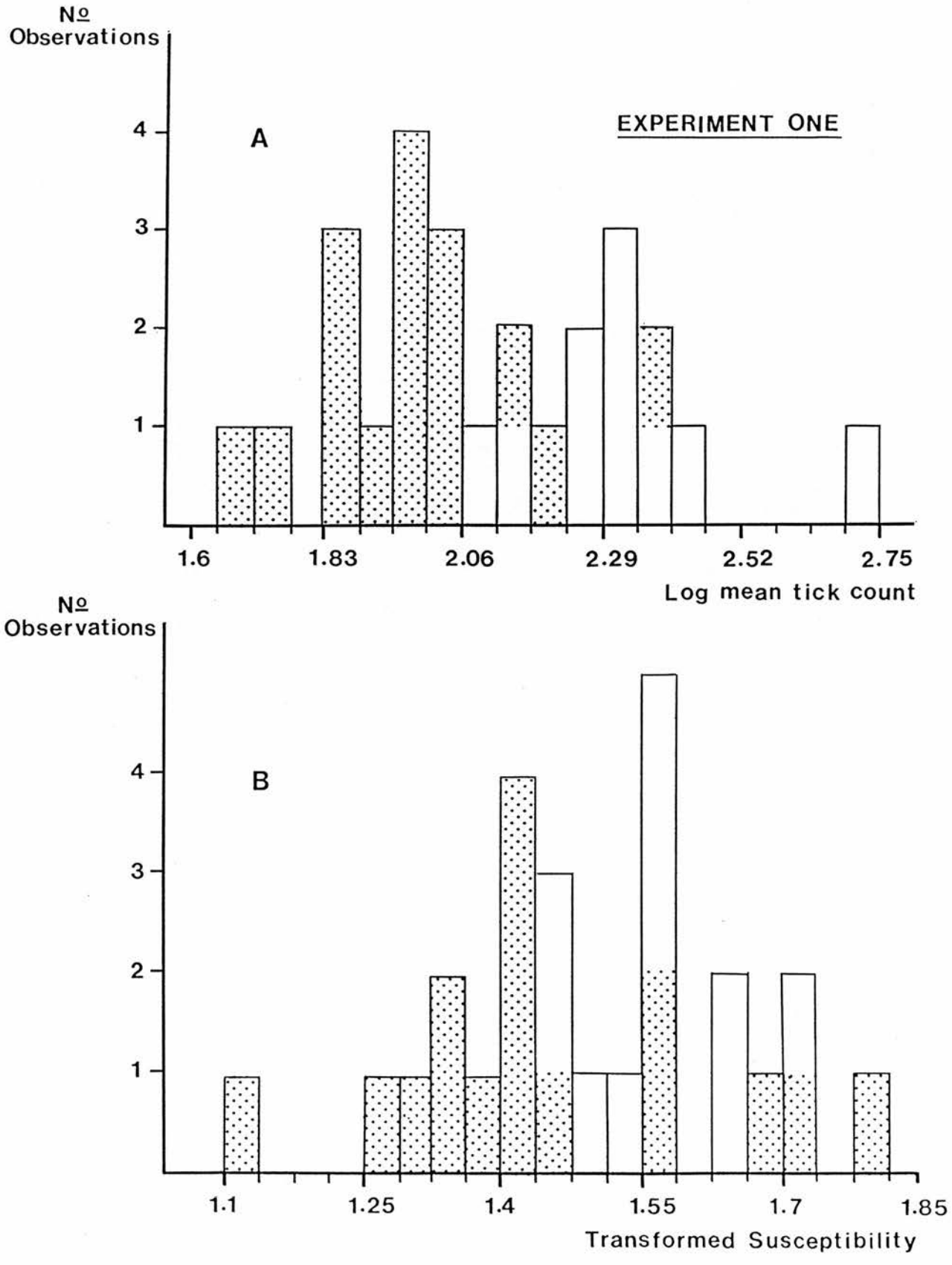
b) Experiment two (Holstein = 15 crossbred = 16)

Antigen @	Holstein	Crossbred	"t" Statistic	P
P1	27.6	15	3.344*	0.002
P3	31.4	23.5	1.536	0.139
P4	43.9	39.9	0.945	0.352
P5	39.9	40.1	0.046	0.963
P6	38.1	34.0	0.991	0.333
D2	36.5	33.5	0.604	0.551
GSG	33.8	37.5	0.821	0.419

@ : P1, P2, P3, P4, P5, P6 and D2 = antigens purified by anion-exchange chromatography from larvae. GSG = Salivary gland homogenate from tick females. Experiment one and two used materials obtained after different chromatographic separations.

* : Indicates dissimilar variances on both groups and use of a modified "t" test.

Figure 8.3. Distribution of counts of semi-engorged Boophilus microplus female ticks (length 4.5-8.0 mm) in two groups of cattle exposed to the tick under natural grazing conditions (A), and distribution of tick counts performed on the same animals after artificial infestation with 10,000 larvae (B). Counts resulting from field infestations are presented as the mean of four counts (transformed to logarithmic scale). Counts from the artificial infestation were performed three weeks after applying the larvae and are presented as percentages of female ticks surviving to maturity (percentages were transformed by exponentiation to 0.25 to normalize the distribution). Shaded areas in the bars indicate the distribution of the values for the Holstein group of animals (n=16). Unshaded areas in the bars show the distribution for the crossbred group (n=10).


Nº Observations
A
EXPERIMENT ONE
4
3
2
1
1.6
1.83
2.06
2.29
2.52
2.75
Log mean tick count
Nº Observations
B
4
3
2
1
1.1
1.25
1.4
1.55
1.7
1.85
Transformed Susceptibility

presented on the original scale). Log mean counts of ticks resulting from field infestations ranged from 1.7 to 2.73 (figure 8.3A). Groups of animals differed in their mean levels of infestation (Holstein = 1.98, crossbred = 2.32). The correlation between the susceptibility to the tick and the log mean count from field infestations was highly significant ($r = 0.60$, $P < 0.01$).

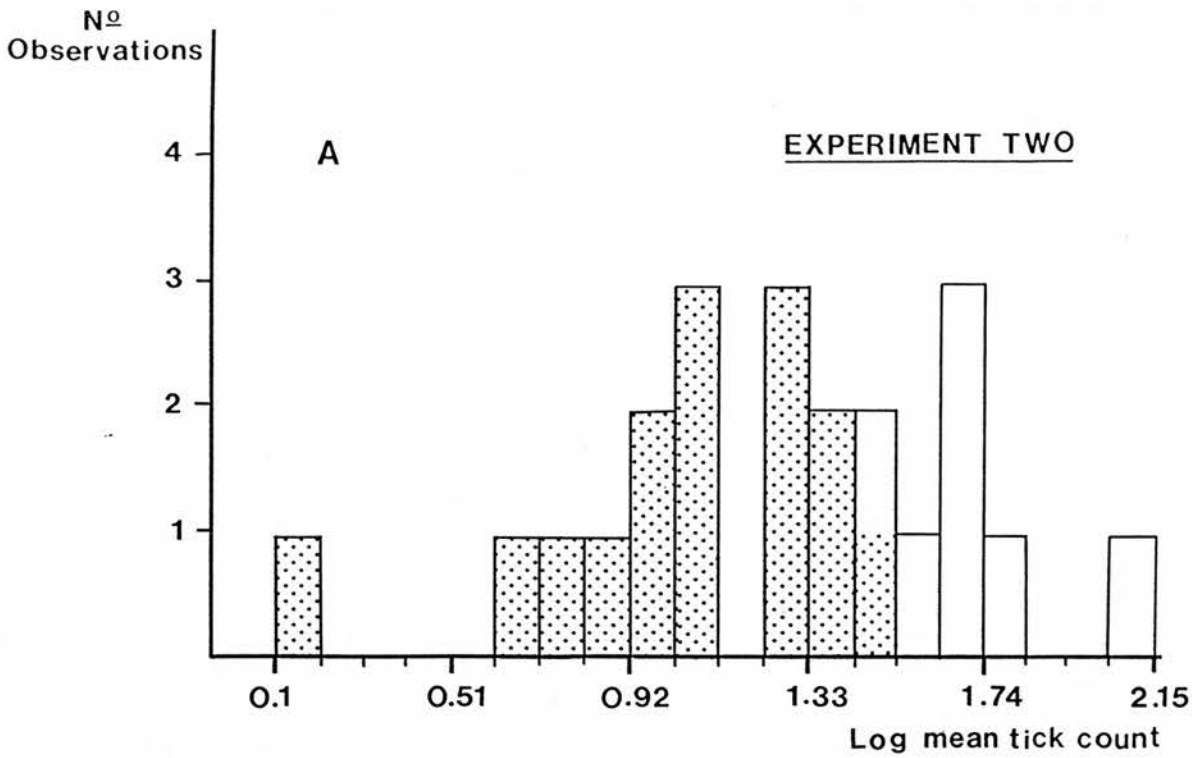
In experiment two, the susceptibility to the tick ranged from 1.2% to 28.2% (figure 8.4B). Both groups of animals showed similar variances and their means were significantly different ($P < 0.001$). Means were: Holsteins = 16.4%, crossbred = 3.2%. Log mean counts of ticks from field infestation ranged from 0.2 to 2.14 (figure 8.4A). The mean for the Holstein group, 1.04 was highly significantly different ($P < 0.001$) from the mean of the crossbred group (1.72). A negative correlation between field counts and the artificial infestation results was found ($r = -0.50$, $P < 0.05$). Field counts were not performed on nine of the animals of the crossbred group during this experiment for the above mentioned restrictions for experimental use.

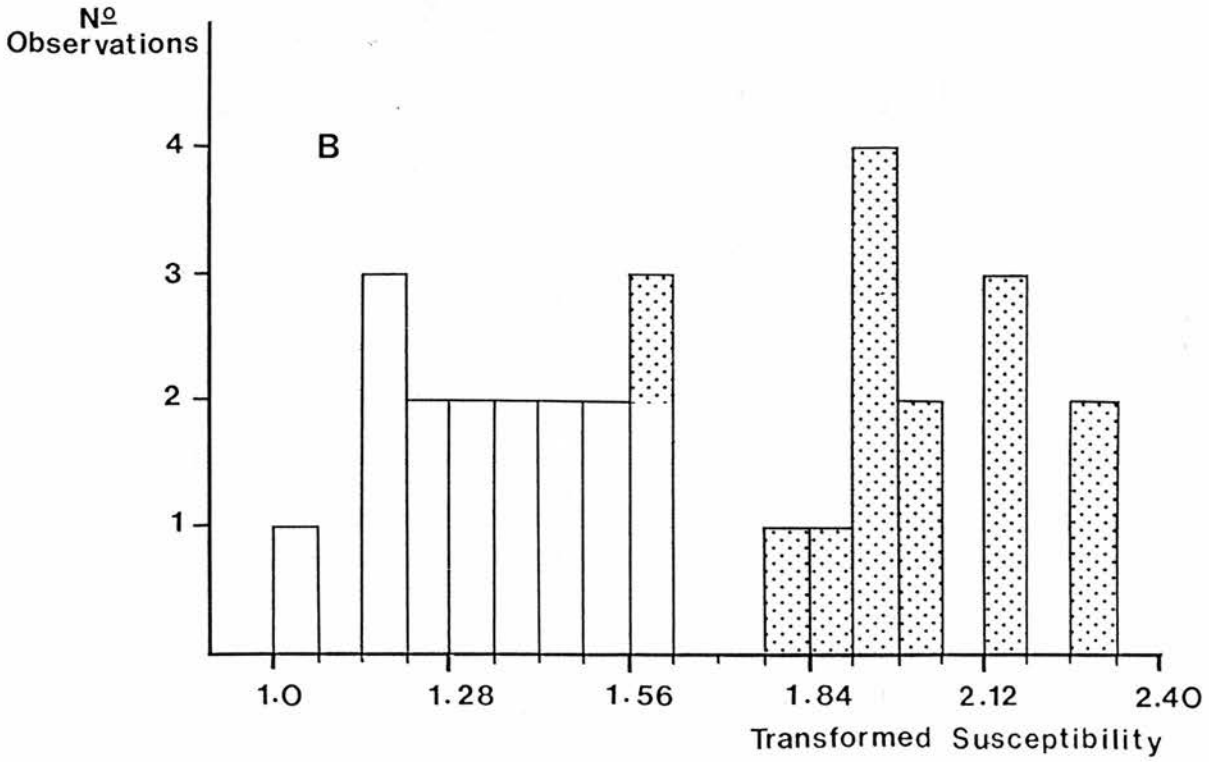
Twelve of the Holstein calves were used in both experiment one and experiment two. No correlation was found on results from the two experiments on the values of susceptibility to the tick ($r = 0.29$, $P > 0.10$), or on results from field tick counts ($r = 0.38$, $P > 0.10$).

8.3.3.- Relationship between skin test responses and the susceptibility to the tick.

A multiple matrix of correlations was prepared in a similar way

Figure 8.4. Histogram of frequencies of the counts of semi-engorged Boophilus microplus female ticks (length 4.5-8.0 mm) in two groups of cattle exposed exposed to the tick under natural grazing conditions (A), and histogram of frequencies of tick counts performed on the same animals after artificial infestation with 10,000 larvae (B). Counts resulting from field infestations are presented as the mean of three counts (transformed to logarithmic scale). Semi-engorged females were counted three weeks after the artificial infestation and are presented as percentages of female ticks surviving to maturity (percentages were transformed by exponentiation to 0.25 to normalize the distribution). Shaded areas in the bars indicate the distribution of values for the Holstein group of animals (n=15) and unshaded areas in the bars show the distribution of values for the crossbred group. Artificial infestation was not performed on a Holstein animal. Only 7 animals of the crossbred group were evaluated for field infestation, but all of this group (n=16) were evaluated by the artificial method.


Nº
Observations
A
EXPERIMENT TWO
4
3
2
1
0.1
0.51
0.92
1.33
1.74
2.15
Log mean tick count


Nº
Observations
B
4
3
2
1
1.0
1.28
1.56
1.84
2.12
2.40
Transformed Susceptibility

as described in previous chapters (5.2.3). In each experiment reactions elicited by each antigen, measured as the diameter of the reaction at 30 minutes post injection or the compiled reaction at 4 and 24 HPI, were correlated with each of the two measures of the tick levels on the animals, the field counts or the evaluation by artificial infestation. For the analysis, data were grouped on different arrays: the totality of the animals, Holstein group only or crossbred group only.

Complex patterns of associations were found in both experiments. The results are illustrated in table 8.3 for experiment one and in table 8.4 for experiment two. Only parameters showing significant correlation coeficients are presented.

In experiment one, when values from the totality of the animals were included in the correlation analysis, significant negative correlations ($P < 0.05$) were observed between the parameter transformed susceptibility (obtained by tick counts from artificial infestation) and reactions to antigens P3, P6 and GSG (diameter reaction at 30 minutes for the former and compiled reactions at 24 HPI for P6 and GSG). However, this association was not found when the log mean count from field infestation were used for the analysis. When data were correlated by groups according the breed type of the animals, it was seen that the correlation pattern differed between Holsteins and crossbred animals. In general, stronger correlations were observed in the crossbred group using both tick counts from artificial or field infestations (table 8.3).

In experiment two a similar and even more contradictory pattern was found. For example, using the totality of animals for the analysis, the compiled reaction at 4 hours to the antigen P1, was

Table 8.3. Differences in the correlation coefficients according to the type of animals evaluated when the relationship between the susceptibility to the tick Boophilus microplus and the dermal reactivity to the inoculation of tick-derived antigens was studied in cattle previously exposed to the tick. Susceptibility was measured by counts of semi-engorged females ticks resulting either from field infestations (F) or from artificial infestations with larvae (A). Experiment one, using antigens obtained after chromatography of the larval extract L2-65.

Skin Test Parameter 1	Total Animals n = 26	Holstein Group n = 16	Crossbred Group n = 10	Tick count 2
Compiled at	-0.33	-0.31	-0.66*	A
4 h. to P1	-0.07	-0.24	-0.07	F
Diameter at	-0.26	-0.08	-0.83**	A
30 min to P2	-0.16	0.01	-0.40	F
Compiled at	0.03	0.15	-0.51	A
4 h. to P2	-0.02	0,18	-0.67*	F
Diameter at	-0.41*	-0.23	-0.87**	A
30 min to P3	-0.17	0.19	-0.35	F
Compiled at	-0.47*	-0.55*	-0.25	A
24 h. to P6	-0.25	-0.24	-0.33	F
Compiled at	-0.02	-0.08	-0.05	A
4 h. to GSG	-0.03	0.05	-0.89***	F
Compiled at	-0.43*	-0.60*	-0.57	A
24 h. to GSG	-0.20	-0.37	-0.56	F

* = (P < 0.05), ** = (P < 0.01), *** = (P < 0.001)
1 : Compiled parameter = Diameter x increase in thickness (mm).
2 : A = Correlation using values of susceptibility obtained by artificial infestation with 10,000 larvae.
F = Correlation using data from field counts (log mean).

Table 8.4. Comparison of the correlation coefficients observed in Holstein and crossbred animals when the susceptibility to the tick Boophilus microplus was correlated with the dermal reactivity to the inoculation of tick derived antigens in animals previously exposed to the tick. Experiment two, using antigens obtained after chromatography of the larval extract L4-65.

Skin Test parameter 1	Total animals 2	Holstein group 2	Crossbred group 2	Tick count 3
Compiled at	0.54** (22)	0.33 (14)	-0.28 (14)	A
4 h. to P1	-0.53* (22)	-0.21 (15)	-0.51 (7)	F
Diameter at	0.20 (21)	-0.88** (7)	0.09 (14)	A
30 min to P3	-0.64* (14)	-0.45 (7)	0.05 (7)	F
Compiled at	-0.17 (21)	-0.46 (7)	-0.68** (14)	A
4h. to P3	-0.65* (14)	-0.16 (7)	-0.34 (7)	F
Diameter at	0.06 (27)	-0.24 (14)	-0.10 (13)	A
30 min to P4	-0.72** (21)	-0.71** (15)	0.34 (6)	F
Compiled at	0.35 (27)	0.02 (14)	-0.11 (13)	A
4 h. to P4	-0.73** (21)	-0.67** (15)	-0.40 (6)	F
Diameter at	0.08 (28)	0.02 (14)	-0.13 (14)	A
30 min to P6	-0.64** (22)	-0.52* (15)	0.22 (7)	F
Compiled at	0.01 (28)	0.04 (14)	-0.53* (14)	A
4 h. to P6	-0.51* (22)	-0.62* (15)	0.01 (7)	F
Compiled at	0.21 (27)	0.14 (14)	-0.29 (13)	A
4 h. to D2	-0.72***(21)	-0.62* (15)	0.34 (6)	F
Compiled at	0.02 (27)	0.03 (13)	-0.64* (14)	A
4 h. to GSG	-0.48* (21)	-0.50 (14)	0.07 (3)	F

* = ($P < 0.05$), ** = ($P < 0.01$), *** = ($P < 0.001$)

1 : Compiled parameter = diameter x increase in thickness.

2 : Figures in brackets indicate number of animals included in the correlation.

3 : A = Correlation using values of susceptibility obtained by artificial infestation with 10,000 larvae.
F = Correlation using data from field counts (log mean).

positively correlated ($r = 0.54$, $P < 0.01$) with the parameter transformed susceptibility, but negatively ($r = -0.53$, $P < 0.05$) with the log mean count. When data of each group of animals were analysed independently, significant negative correlations were found between reactions to antigens P3, P4, P6, D2 and GSG and any of the two measures of the susceptibility to the tick. This time as opposed to experiment one, in general stronger correlations were observed on the Holstein group.

Information on susceptibility of the animals to the tick was then compiled using a ranking approach. In each experiment, and within each group of animals, individuals were ranked independently on the two parameters (field or artificial infestations), and a Spearman rank correlation coefficient between the two measures was calculated (Siegel, 1956). Then the individual ranks were summed and a new rank was allocated on those produced values and named as susceptibility rank. In experiment two, where missing values were present on one of the two parameters, the rank obtained for the animal in the other parameter was doubled to calculate the susceptibility rank. This rank was used to calculate its correlation with the results on the skin test at 30 minutes post injection, using Spearman rank correlation coefficients.

In experiment one, the non-parametric correlation between the two measures of susceptibility to the tick was significant in the Holstein group ($r' = 0.518$, $P < 0.025$), but not in the crossbreds ($r' = 0.503$, $P > 0.10$). In experiment two, no significant association was observed on the Holstein group ($r' = 0.442$, $P > 0.10$), but a significant association was observed in the crossbreds ($r' = 0.714$, $P < 0.05$).

Tables 8.5 and 8.6 present the production of the susceptibility rank respectively for experiment one and for experiment two. In general, the animals located at any extreme of the distribution were similary ranked by the tick counts either from artificial or field infestations, (animals 8539 and 8567 on experiment one for example). Under this arrangement, rankings obtained for the Holstein calves used in both experiments were compared. The non-parametric correlation coefficient was nearly significant ($r' = 0.498$, $P < 0.10$, > 0.05).

In experiment one, non-parametric associations between the susceptibility rank and the diameter of reaction at 30 minutes were observed. In the Holstein group, correlations were found with antigens P1 ($r' = -0.365$, $P < 0.10$, > 0.05) and P4 ($r' = 0.461$, $P < 0.05$). However, the association with P4 was a positive one. In the crossbred group significant correlations were found with antigens P2 ($r' = -0.728$, $P < 0.05$) and P3 ($r' = -0.776$, $P < 0.01$).

In experiment two, non-parametric associations in the Holstein group were observed to antigens P3 ($r' = -0.561$, $P < 0.10$, > 0.05), P4 ($r' = -0.493$, $P < 0.05$), P6 ($r' = -0.377$, $P < 0.10$, > 0.05), D2 ($r' = -0.392$, $P < 0.10$, > 0.05) and GSG ($r' = -0.456$, $P < 0.05$). In the crossbred group, weaker positive correlations were observed to antigens P4 ($r' = 0.398$, $P < 0.10$, > 0.05), D2 ($r' = 0.381$, $P < 0.10$, > 0.05) and GSG ($r' = 0.360$, $P < 0.10$, > 0.05).

Due to the inconsistency of the results in both experiments and with the different breed type of the animals, the relationship was studied by a graphic plot. In each experiment and within each group

Table 8.5. Susceptibility to the tick Boophilus microplus in Holstein and crossbred animals with wide previous exposure to the tick, measured by counts of semi-engorged females resulting either from field or artificial infestations and arranged in ranks. Experiment one. La Libertad 1987.

a) Holstein group

Animal number	Artificial infestation rank	Field infestation rank	Summed ranks	Susceptibility Rank	Category *
8515	7	5	12	4.5	R
8519	2	3	5	2	R
8525	1	13	17	9	S
8531	12	10	22	12	S
8535	13	14	27	14	S
8539	1	2	3	1	R
8545	5	8	13	6	S
8557	14	15	29	15	S
8563	8	12	20	10.5	S
8565	9	11	20	10.5	S
8567	15	16	31	16	S
8569	10	6	16	8	S
8573	3	4	7	3	R
8575	6	9	15	7	S
8577	16	7	23	13	S
8597	11	1	12	4.5	R

b) Crossbred group

Animal number	Artificial infestation rank	Field infestation rank	Summed ranks	Susceptibility Rank	Category *
5347	1	1	2	1	R
6313	5	7	12	6	S
6323	2	2	4	2	R
6327	3	10	13	7	S
6333	10	9	19	10	S
6337	4	5	9	3.5	S
6343	9	6	15	8	S
6349	7	4	11	5	S
6351	6	3	9	3.5	S
6395	8	8	16	9	S

* : R = Resistant, animals on the 1/3 lower ranks within each group
S = Susceptible, the rest of the animals.

Table 8.6. Rank of the susceptibility to the tick Boophilus microplus in Holstein and crossbred cattle with wide previous exposure to the tick, measured by counts of semi-engorged females resulting either from field or artificial infestations. Experiment two. La Libertad 1987.

a) Holstein group

Animal number	Artificial infestation rank	Field infestation rank	Summed ranks @	Susceptibility Rank	Category *
8400	10	9	19	11	S
8515	-	1	2	1	R
8525	9	8	17	8	S
8531	2	2	4	2	R
8535	11	14	25	14	S
8539	5	6	11	6	S
8545	6	15	21	12	S
8549	14	13	27	15	S
8557	13	5	18	9.5	S
8563	12	11	23	13	S
8569	4	12	16	7	S
8573	7	3	10	4.5	R
8575	3	7	10	4.5	R
8585	8	10	18	9.5	S
8597	1	4	5	3	R

b) Crossbred group

Animal number	Artificial infestation rank	Field infestation rank	Summed ranks @	Susceptibility Rank	Category *
5321	11	-	22	14	S
5325	14	-	28	16	S
5371	7	-	14	9.5	S
5379	2	-	4	2	R
5381	5	-	10	5	R
5385	8	-	16	11	S
5397	1	-	2	1	R
5405	6	-	12	7	S
6321	16	7	23	15	S
6367	13	5	18	12	S
6369	15	6	21	13	S
6375	12	2	14	9.5	S
6385	9	4	13	8	S
6389	3	3	6	3	R
6511	4	-	8	4	R
7301	10	1	11	6	S

* : R = Resistant, animals on the 1/3 lower ranks within each group.
S = Susceptible, animals on the 2/3 upper ranks.
@ : When only rank in one parameter was available it was doubled.

of animals, individuals located in the third lower ranks were assigned to the category R (resistant) and the two thirds upper ranks given the category S (susceptible). Frequency histograms of the diameters of the reactions were plotted for each of the antigens and on them, the values obtained by the animals categorized as R were marked. Results for experiment one are presented in figure 8.5 where values obtained for the tick naive animals were also recorded. The results for experiment two are presented in figure 8.6.

In experiment one, individuals categorized as R from both groups tended to be located on the upper half of the distribution for P1, P2 and P3 but differences in the reactivity to different antigens were observed according to the breed type of the animals (On P4 for example in figure 8.5). It can also be observed in figure 8.5, that the tick naive calves displayed minimum reactivity to all the antigens. This supports the assumption that reactions observed on the tick exposed animals are immune mediated.

In experiment two, the discriminative efficiency of the skin test was less evident, particularly on the crossbred group, where one animal (number 6389) showed minimum reactions to all antigens. However, all Holstein animals categorized as R for resistant, were located on the upper half of the distribution of the reactions to P3.

8.4.- DISCUSSION.

Susceptibility to the tick as measured by artificial infestations showed differences between the Holstein and the crossbred cattle in both experiments, but in an inconsistent

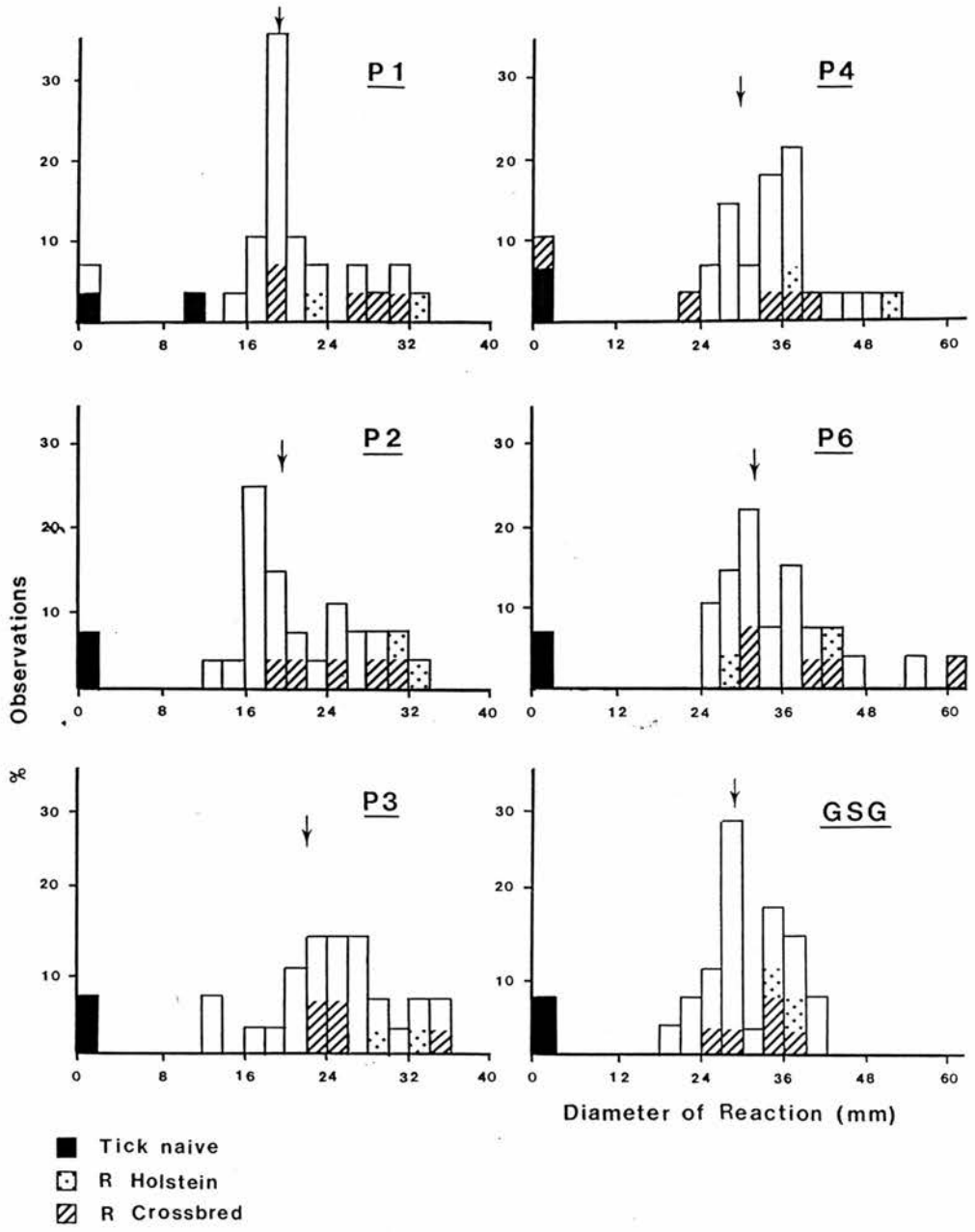


Figure 8.5. Comparison of the discriminative efficiency of a skin test to detect cattle which display resistance to the tick Boophilus microplus. The skin test used antigens separated by anion-exchange chromatography from the tick larval extract L2-65 (P1, P2, P3, P4 and P6) or a salivary gland homogenate from tick females (GSG). Histograms ilustrate the frequency distribution of diameters of the reactions elicited 30 minutes after injection of the different antigens, on 26 animals exposed (16 Holstein, 10 crossbred), and on 2 unexposed to the tick. Values obtained by the third most resistant animals within each breed group are indicated as well as those observed in the tick naive animals. The clear areas in the bars correspond to the values obtained by the susceptible animals. The mean of the parameter for each antigen is indicated by an arrow.

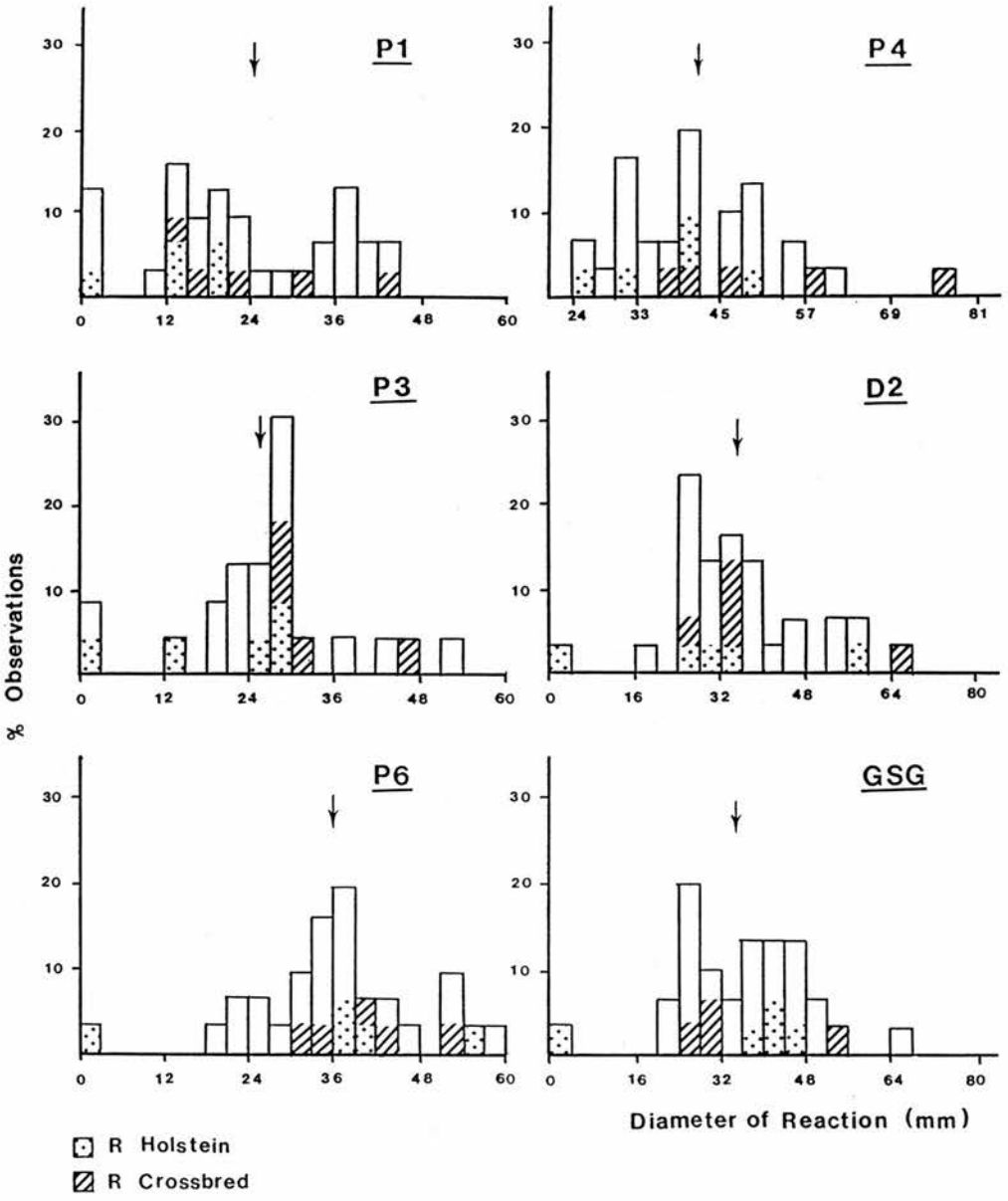


Figure 8.6. Discriminative efficiency of the dermal reactions induced by the injection of different antigens derived from the tick Boophilus microplus, to detect cattle which display resistance to the tick. Antigens P1, P3, P4, P6 and D2 were separated from the larval extract L4-65 by anion-exchange chromatography. GSG is a salivary gland homogenate from tick females. The histograms illustrate the frequency distribution of diameters of the reactions, 30 minutes after injection of the various antigens on Holstein and crossbred cattle previously exposed to the tick. Reactions displayed for the third most resistant animals within each breed group are indicated with different patterns. The clear areas in the bars correspond to the values obtained by the susceptible animals. The arrows point the location of the mean for each antigen.

pattern. The low susceptibility found in the Holstein group in experiment one has already been discussed (6.4). When the artificial infestation was conducted in January 1988, the values of susceptibility were similar to those observed in the same animals when evaluated during November and December 1986 (6.3.2). This illustrates the instability of the resistance to the tick in this group of animals.

Crossbred animals used in experiment one and two were two different groups of animals, which limits the comparisons between both experiments. However, their means were similar, 6.0% in experiment one and 3.2% in experiment two. These values were lower than those observed in the Holstein group in experiment two (16.4%), which is explained by their zebu content (Seifert, 1971).

Tick counts resulting from field infestations are difficult to compare since both groups of animals were grazed on different pastures before the counts. However, they are a good indication of the tick burdens experienced for the animals before the skin test. In experiment one (July-September 1987) these were higher for the crossbred than for the Holsteins. However, it should be noted that after the count performed in July, the animals experienced high tick burdens in spite of the acaricide treatments, which precluded the performance of the artificial infestation (table 8.1).

Tick counts on the Holstein group were conducted six weeks after the last application of acaricide. However, the number of ticks on the animals was reduced by the effect of those treatments on the whole population of ticks on the pasture (Norton, Sutherst and Maywald, 1983). This fact may have caused the negative correlation found between field and artificial counts. When values

from both groups of animals were analysed separately, the relationship was positive in both breed groups and significant in the crossbred group (as detected by the non-parametric correlation).

The relationship between susceptibility to the tick and results of the skin test is affected by the same factors as discussed above. Correlations calculated using the totality of the animals are then considered to be meaningless and are presented only to illustrate the dangers of drawing conclusions based on tick counts performed on animals managed under dissimilar conditions.

It was expected that tick counts based on artificial infestations would provide a more accurate estimation of the relative susceptibility of each animal to the tick. On the other hand, tick counts from field infestations were performed nearer the time of the skin test, minimizing any enviromentally related change in the resistance status of each animal (Bennett and Wharton, 1968; Seifert, 1984) and so in the measure of their association with the skin test.

The lack of agreement in the correlation coefficients of that relationship, calculated using either field or artificial counts, explains the low association between the two measures of susceptibility to the tick. However, differences in the pattern of correlation with the skin test in the two groups of animals were not expected.

Experimental error in the estimation of the levels of susceptibility to the tick, based on the artificial infestations, could not be excluded since the animals were grazed on a not entirely tick free pasture. However, the results are comparable with

groups of animals subject to similar managerial procedures (Seifert, 1984).

The ranking approach was therefore considered in an attempt to summarize the results obtained by the two methods of measuring susceptibility to the tick. No significant correlation was observed in the rankings of susceptibility obtained by the 12 Holstein calves used in both experiments. However, with the exemption of calf 8531 all calves were assigned to similar lower or higher ranks. For example calves 8515, 8573 and 8597 were allocated on the R category in both experiments.

Non-parametric significant correlations were observed between results in the skin test and the ranking in susceptibility to the tick, but again different patterns were observed on both groups of animals in the two experiments. The visual plot demonstrated that in experiment one, the animals categorized as R were located in the upper half of the distribution of the reactions to antigens P1, P2 and P3 without distinctions on the breed type. In experiment two, this discriminative efficiency of the skin test was only reproduced in reactions to the antigen P3 in the Holstein group. It should be noted however, that in experiment two, reactions to the antigen P3 showed significant parametric correlations with the susceptibility to the tick (measured by artificial infestations) but at different times; at 30 minutes for the Holstein group and at four HPI for the crossbreds. In this way P3 constitutes a potential antigen for subsequent studies.

Finally, the inconsistency in the correlations found in the Holstein and in the crossbred groups during experiment two and the differences in the time of reading the reactions in which they were

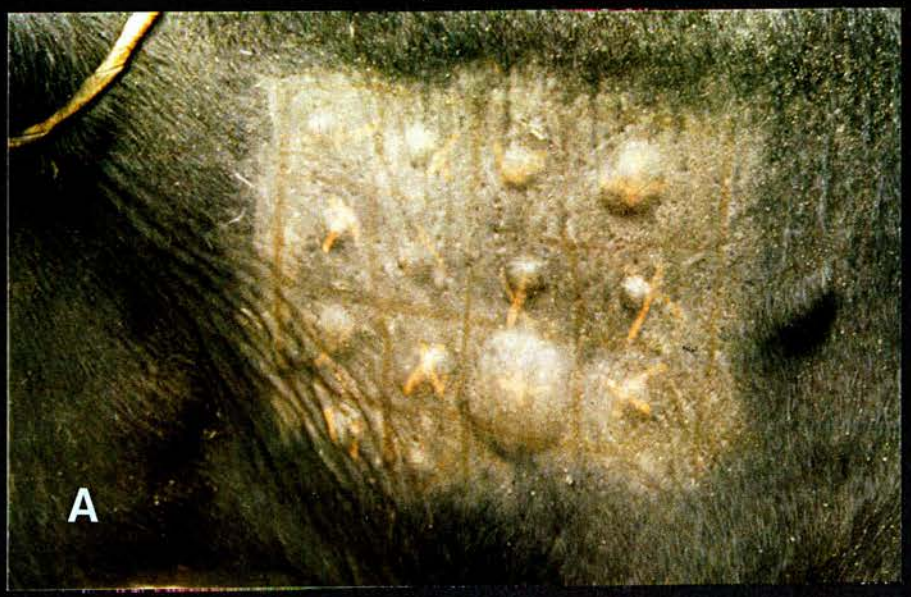
associated with the susceptibility to the tick, seem to be due to differences in the thickness of the normal skin in each group of animals.

Figure 8.7 illustrates the difficulties found in measuring the reactions in animals of different skin thickness. The Holstein animals had relatively thin skin (mean skin fold thickness and standard error on experiment two: 5.4 $\pm$ 0.31 mm), which was significantly different ($P < 0.01$) from the mean of the crossbred (7.9 $\pm$ 0.63 mm), in addition some individuals of the latter group displayed very thick skins, which made the reading of the reactions difficult. For example, animal 6389 which showed minimum reactivity to all antigens during experiment two and was categorized as R, had a mean skin thickness of 12.5 mm. This is an important factor which must be taken into account when designing the methodology to measure the skin reactions in this type of animal. It is probable that the measure of the increase in thickness produced by the hypersensitivity reaction at 4 HPI (because at 30 minutes reactions are painful and not indurated) will be a more accurate measure of the reactions in thick skinned cattle.

8.5.- CONCLUSIONS.

- All antigens used in these two experiments, at the injected concentrations, elicited immediate hypersensitivity reactions in both Holstein and crossbred cattle previously exposed to the tick. Reactions displayed maximum size at 30 minutes post injection.
- Susceptibility to the tick was assessed in the animals by

Figure 8.7. Measurement of dermal hypersensitivity reactions to tick derived antigens in cattle with differing degrees of resistance to the tick Boophilus microplus. The difficulties involved in the measuring of the reactions in different breeds of cattle are illustrated. Plate A shows typical hypersensitivity reactions on a thin skinned Holstein animal. Reactions are easily visualized and delimited. Plate B shows hypersensitivity reactions on a crossbred animal. They are noticeable but not easily delimited on the areas of clear skin. Plate C demonstrates reactions on a crossbred thick skinned animal. They are palpated rather than assessed visually.


A


B


C

counts of semi-engorged females resulting either from field or from artificial infestations. Different levels of susceptibility were observed between Holsteins and crossbred cattle in both experiments, but not in a consistent pattern. Various factors appear to have caused this variability, including: instability of the resistance in the Holstein group of calves, experimental error related with the artificial infestation being performed on tick contaminated pastures and the fact that tick counts from field infestations were carried on cattle grazed on different pastures and subjected to dissimilar managerial procedures. It is considered that these factors should be kept constant when analysing susceptibility to the tick in groups of animals.

- The relationship between the responses in the skin tests using different antigens and the susceptibility to the tick was studied independently in each group of cattle and in each experiment. Both the parametric correlation with the two susceptibility parameters described above, and a non-parametric correlation with a rank of susceptibility produced using a combination of the ranks attained for each individual in each parameter were used. A visual plot of the skin responses displayed by the less susceptible animals in each group to the different antigens was also used. Using that rationale it was concluded that the antigen P3 was a candidate for next set of experiments studying that relationship.

- It was suggested that the thickness of the normal skin influenced the accuracy of the measurement of the reactions and hence the strength of the relationship with the susceptibility

to the tick. This is a factor which must be taken into account in the design of the methodology to measure the skin reactions in the following experiments.

CHAPTER NINE:

COMPARISON OF THREE METHODS TO EVALUATE THE RESISTANCE TO Boophilus microplus ON CROSSBRED CATTLE UNDER FIELD CONDITIONS

SUMMARY

When evaluated repeatedly at five week intervals, a group of 33 cattle grazed under tropical conditions displayed changes in their level of susceptibility to the tick Boophilus microplus. Higher levels of infestation with ticks were in July and August (middle of the rainy season in the area of study). The animals were similarly ranked for tick counts in all the evaluations. Skin test reactivity to the inoculation of tick-derived antigens and antibody titres to salivary gland antigens from the tick were also measured. Lower levels of reactivity in the skin test and higher antibody titres were observed on July, when significant negative correlations of these responses with the tick count of the animals were observed. It was suggested that in times of heavy tick challenge, animals of high susceptibility to the parasite experience a suppression in the skin test reactivity, which was associated with the presence of high titres of specific immunoglobulin G antibodies.

9.1.- INTRODUCTION.

In the previous chapters, various experiments to study the relationship between the level of resistance to the tick Boophilus microplus and the dermal responsiveness to the inoculation of tick derived antigens in animals with differing levels of resistance,

have been presented. In those experiments, various factors have been stated to affect both the level of resistance, the size of the reactions on the skin test and the measure of that relationship. Some of these factors are related to management: nutrition, instability of the resistance (6.4) and the contamination of the pastures with ticks which decreases the reliability of the measure of resistance (8.4). Other factors discussed have been related to the ways in which data was presented for statistical analysis (6.4, 8.4), and the design of the experiment (7.4).

As the final stage during this series of studies, the experiment presented here was designed to reduce the variability in the responses of both the resistance to the tick and the skin test reactivity and at the same time test the repeatibility of the measurements performed on the same animals at equivalent time intervals. To accomplish this, a group of cattle with wide previous exposure to the tick was tested four times for both levels of resistance to the tick and skin reactivity to semi-purified tick derived antigens. Animals were grazed on an experimental paddock at La Libertad research station where B. microplus ticks are present on the pastures throughout the year.

9.2.- MATERIALS AND METHODS.

9.2.1.- Experimental animals and pastures.

Two groups of animals were allocated for experimental use on a permanent basis (as opposed to the previous experiment). A group of 18 crossbred Gyr-Holstein heifers, 16 to 20 month old and another

group of 15 crossbred castrated males 2 to 3 years old.

The Gyr-Holstein group had 50% Zebu (Bos indicus) content and were born at the Tibaitatá research centre, located in the tick free area of the country. Some of them were used in a previously described experiment (7.2.1). They were moved to La Libertad research centre located in the tick infested area of the country in March and April 1988. These animals were premunized to Babesia bovis, Babesia bigemina and Anaplasma marginale clinical infection by intravenous injection of attenuated organisms (B. bovis) or by a controlled exposure to ticks and prophylactic treatment with 20 mg/kg of oxytetracycline (Terramicina L.A., Pfizer, Colombia) and 2 mg/kg of imidocarb (Imizol, Coopers, Colombia). The animals were allowed to graze on tick infested pastures until the experiment was commenced.

The second group of animals were of a complex mixture of breeds (hybrids and trihybrids) including Bos indicus (Brahman) and Bos taurus breeds both European (Red Holstein, Jersey, Brown Swiss and Normande) or Criollo types (San Martinero and Blanco Orejinegro). These animals were born in the tick infested area and had experienced natural tick infestations throughout their lives.

A 20 hectares paddock of the grass Brachiaria decumbens was used to maintain all the animals during the experiment. The pasture experienced changing levels of larval tick contamination during the study (9.2). Mineral salt ad libitum was the only supplement offered to the cattle. All animals were subjected to identical management procedures.

9.2.2.- Experimental design.

Four skin tests and four estimations of the susceptibility to the tick were conducted on all the animals. In each repeat, the animals were artificially infested with larvae the day on which the skin tests on all the animals had been finished. Tick counts were performed three weeks later during five days, and the following skin test was conducted two weeks after the tick count. Sera for analysis of anti-tick antibodies was collected at the same time of the skin test performance. The level of contamination of the pastures by tick larvae was estimated by flagging the week previous to the artificial infestation.

Due to excessive tick burdens on the animals and on the pastures, the second artificial infestation was not conducted and tick numbers resulting from field infestations were assessed instead. Then the animals were sprayed with deltametrine (Bayticol, Bayer Colombia). In subsequent tests, animals were sprayed with coumaphos (Asuntol, Bayer Colombia), the day of the last tick count from the artificial infestation were counted. The final chronology of the experiment is displayed in table 9.1.

9.2.3.- Tick counts.

9.2.3.1.- Tick counts from artificial and field infestations of the animals.

At the end of each skin test (with the exception of the second test), each animal was artificially infested with 20,000 B.microplus larvae using two phials of 0.5 grams of eggs each from the laboratory colony maintained at La Libertad. Methods for infestation

Table 9.1. Chronology of the experiment "comparison of three methods to evaluate the resistance to Boophilus microplus in crossbred cattle under field conditions".

Date	Week	Activity
2/06/88	1	Coumaphos spray.
14-18/06/88	3	Estimation of larval levels on pastures.
20-22/06/88	4	First skin test, collection of sera.
22/06/88	4	Artificial infestation first test.
11-15/07/88	7	Count of ticks first test.
18-22/07/88	8	Estimation of larval levels on pastures.
25-27/07/88	9	Second skin test, collection of sera.
28-29/07/88	9	Count of ticks from field infestation.
29/07/88	9	Deltametrine spray.
22-26/08/88	13	Estimation of larval levels on pastures.
29-31/08/88	14	Third skin test, collection of sera.
31/08/88	14	Artificial infestation third test.
19-23/09/88	17	Count of ticks third test.
23/09/88	17	Coumaphos spray.
26-30/09/88	18	Estimation of larval levels on pastures.
10-12/10/88	20	Fourth skin test.
12/10/88	20	Artificial infestation fourth test.
31/10-4/11/88	23	Counts of ticks fourth test.
4/11/88	23	Coumaphos spray. End experiment.

(3.1.3) were modified so that the larvae were applied individually to each animal with the help of a brush whilst restrained in the cattle crush. Counts of semi-engorged females (larger than 4.5 mm) were performed from days 19 to 23 after the artificial infestation (3.1.3). Susceptibility to the tick was expressed as a percentage of females surviving to maturity. Where parametric statistics were required, the parameter was transformed as already described (5.3.1) to normalize the distribution.

Ticks resulting from field infestations were counted during the second test. Counts of semi-engorged females on two different days were logarithmically transformed (Log_{10} count + 1) and their average used.

9.2.3.2.- Estimation of the level of contamination of the pastures with tick larvae.

The population of tick larvae on the pastures was estimated the week before the performance of the artificial infestation, using a modification of the hinged flag dragging method described by Wilkinson (1961). For this purpose the paddock was split into 16 imaginary divisions (figure 9.1) by placing coloured flags on the fences, at around 45 m intervals. Each division contained 280 imaginary transects separated from the next by a distance of 1 m, for a total of 4480 transects on all the paddock.

Each sampling comprised five days. On each day three or four divisions were sampled. In each division 10 transects were chosen at random using ballots. In each transect the operator held the sampling device (a 50x50 cms white flannelette attached to a wooden

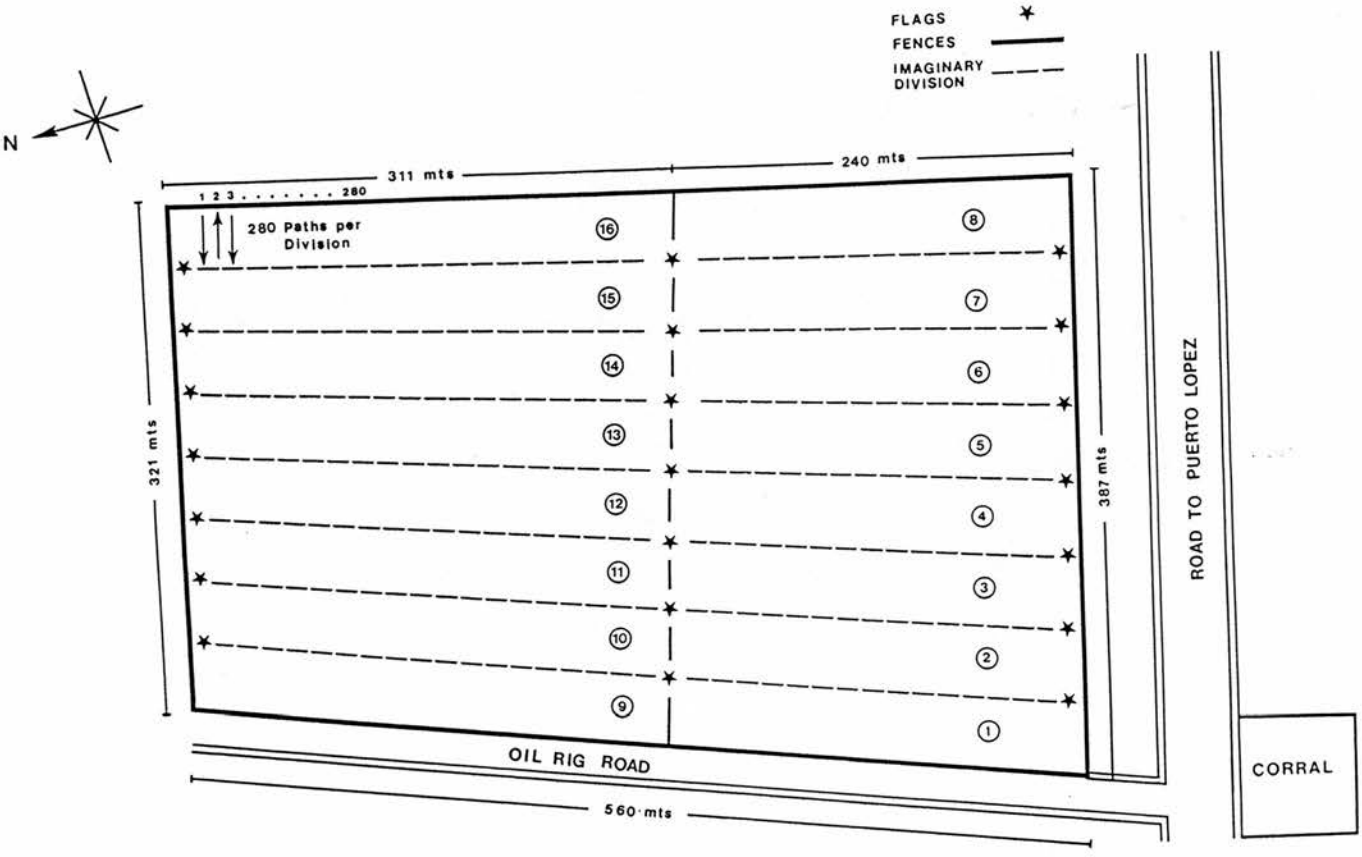


Figure 9.1.- Arrangements to estimate the numbers of Boophilus microplus larvae in an experimental paddock at La Libertad research centre, Villavicencio, Colombia. The paddock was divided by a fence, but animals grazed freely in both divisions. Coloured flags were placed on the fence to produce 16 imaginary divisions of the paddock, each one containing 280 paths or transects of around 40 m length and separated by 1 m. Populations of larvae were estimated by recovery with a sampling device held in front of an operator who dragged it on 3.6% of the transects on a sampling week. The location of the corral where tick counts on the animals were performed, is illustrated.

base) vertically in front of him and walked slowly until the visual intersection of the corresponding flags on the fences. Tick larvae so recovered were counted and returned to the grass with the help of a brush. The procedure was repeated in the same transect in the opposite direction, and the sum of the ticks recovered back and forth were considered as the number of ticks in each transect.

Using this method, 160 transects (3.6%) were sampled each time. Total ticks counted in all transects were considered as an estimate of the population of ticks on the pasture in that particular week. This method had been used extensively at the laboratory in previous years and had proved reliable (Benavides O., E. and Villar, C. unpublished observations).

9.2.4.- Skin test.

Skin tests were conducted using methods previously described (6.2.3, 7.2.4), but only reactions at 30 minutes and 4 HPI (hours post injection) were recorded.

In each test, different antigens were assayed, but P3 and GSG were used in all evaluations. They were chosen in accordance to the partial results obtained in each test, trying to use the most relevant antigens. In all tests each antigen was used at three concentrations (with the exception of GSG in test one used at only the highest concentration): 5, 0.5 and 0.05 micrograms of protein by inoculation site. The following were the antigens used in each test:

test one: P1, P3, P4, P6 and GSG

test two: P3, P5, P6 and GSG

test three: P3, P4, P5 and GSG

test four: P3, P5 and GSG

All antigens were freeze dried and diluted in PBS shortly before the performance of the tests.

9.2.5.- Determination of antibodies to salivary antigens of the tick.

The presence of anti-tick antibodies in the sera of the experimental animals was studied using an ELISA test. Sera obtained before the performance of each skin test, except the fourth, were frozen at -20°C and in November 1988 transported to Edinburgh where the ELISA test was conducted using GSG as antigen. Results are presented as explained previously (7.2.5). The ELISA methodology has also been described (3.6).

9.2.6.- Meteorological records.

Daily information on minimum and maximum environmental temperature, relative humidity and rainfall were collected from the meteorological station at La Libertad research centre. Values of temperature and relative humidity were averaged to produce a weekly value (Thursday to Wednesday). The weekly rainfall consisted of the sum of values from the corresponding week.

9.3.- RESULTS.

9.3.1.- Susceptibility to the tick.

The mean level of susceptibility of the animals to the tick and the larval burdens found on the pasture are presented in figure 9.2.

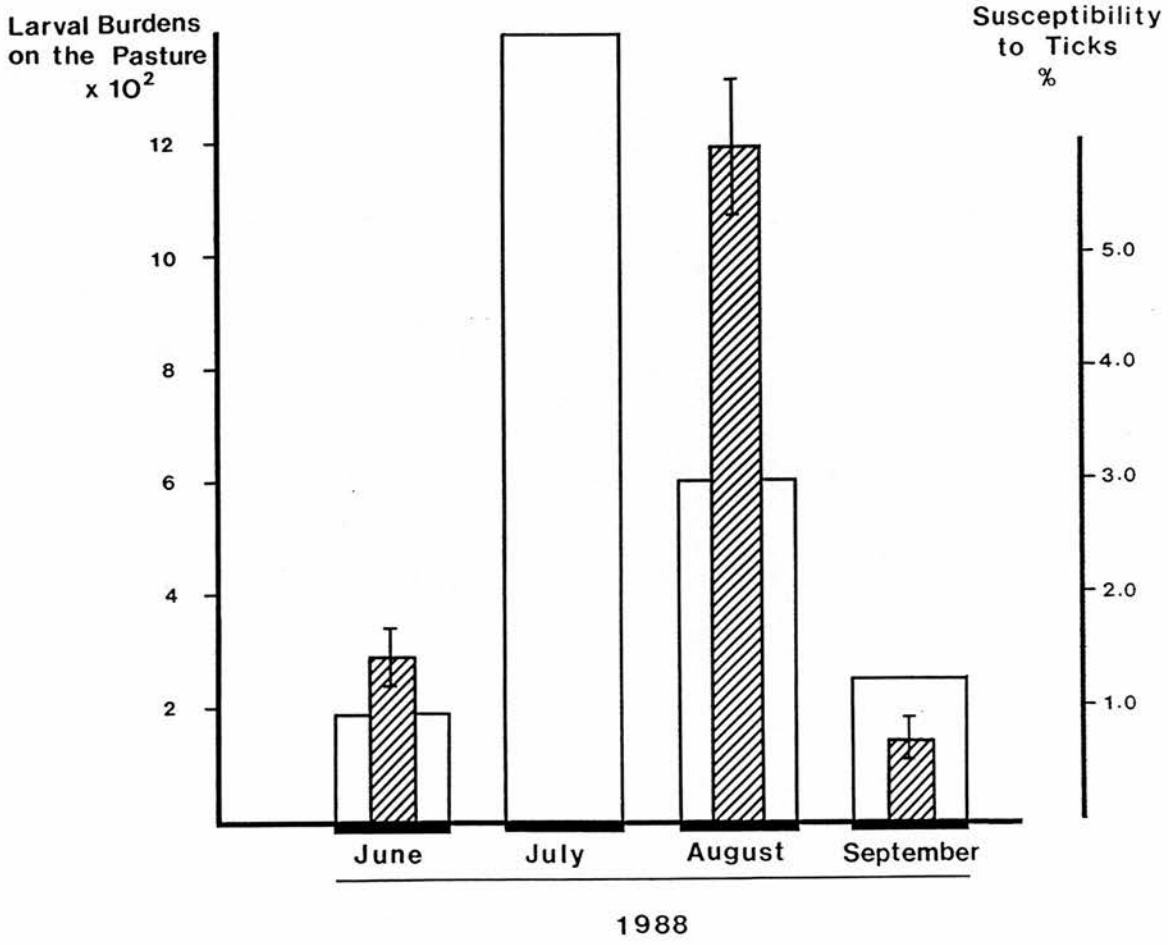


Figure 9.2. Mean levels of susceptibility to the tick Boophilus microplus in 33 cattle grazed under tropical conditions, assessed repeatedly by artificial infestations with 20,000 larvae and expressed as percentages of surviving females (shaded bars). The standard error of each mean is indicated. The clear bars represent the variations in the levels of contamination of the pastures with larvae as estimated by the use of a dragging device the week previous to the artificial infestations.

Susceptibility differed at a significant level ($P < 0.05$) in all the tests (the statistics were calculated using the transformed parameter, but are presented in their original scale). In test one, conducted in June the mean value was 1.44% (range: 0.04-4.63%). In test three conducted in August was 5.94% (range: 1.58-19.4%) and in test four was 0.71% (range: 0.05-16.0%). On the other hand, the estimated mean burden of engorged females resulting from field infestations on July was 111 (range: 12-390).

Recovery of larvae from the pasture using the dragging method were, June = 192, July = 1393 (when the artificial infestation was not conducted due to excessive infestation levels on the animals), August = 601 and September = 254.

Different counts on individual animals were correlated. The correlation coefficients between the mean tick burden from field infestations conducted in July (logarithmically transformed) and the estimation of the susceptibility to the tick on test one (T1), three (T3) and four (T4) (exponentially transformed) were respectively: 0.74, 0.71 and 0.47, all highly significant ($P < 0.01$). Correlations were also found between the three estimates of susceptibility to the tick: (T1/T3: $r = 0.50$, $P < 0.01$; T1/T4: $r = 0.42$, $P < 0.05$; T3/T4: $r = 0.81$ $P < 0.001$).

The tick counts obtained for each animal in each test were ranked and results are displayed in table 9.2. Using an addition of those ranks in the four tests a Kendall's coefficient of concordance (W), was calculated (Siegel, 1956). The coefficient was highly significant ($W = 0.6838$, $P < 0.001$), indicating that the animals were similarly ranked for susceptibility to the tick in all the tests including the count of ticks from field infestations.

Table 9.2. Comparison of the ranks on three estimations of the susceptibility to the tick Boophilus microplus (tests one, three and four), as measured by artificial infestations with 20,000 larvae, and a count of ticks from field infestations (test two) on cattle grazed on an experimental paddock at La Libertad research centre. Villavicencio, Colombia, 1988.

ANIMAL No.	BREED a.	RANKS ON SUSCEPTIBILITY Test one	Test three	Test four	RANK FIELD COUNT	ADDED RANK b.
86389	Sm*Ps.C	1	2	1	1	5.
85405	Sm*C	2	1	2	2	7.
86511	Sm*Bon.J	7	7	6	5	25.
8670	Gyr*H	10	4	7	9	30.
86303	J*C	6	14	5	7	32.
8710	Gyr*H	11	11	16	3	41.
8666	Gyr*H	21	3	10	10	44.
8680	Gyr*H	19	6	13	8	46.
8660	Gyr*H	3	10	14	20	47.
8662	Gyr*H	12	5	18	12	47.
86323	J*C	5	24	15	4	48.
8672	Gyr*H	18	8	11	13	50.
8684	Gyr*H	15	9	4	22	50.
85397	Ps*C.Sm	4	20	17	16	57.
85381	Sm*C	9	19	22	11	61.
8674	Gyr*H	14	13	20	15	62.
86375	Ps*C.Sm	13	12	26	14	65.
8722	Gyr*H	24	16	9	18	67.
8708	Gyr*H	20	18	12	28	78.
8664	Gyr*H	30	15	8	29	82.
8716	Gyr*H	32	17	3	30	82.
8676	Gyr*H	16	27	21	19	83.
8712	Gyr*H	22	21	19	21	83.
85395	N*Ps.C	8	29	31	17	85.
86385	Ps*C	26	26	28	6	86.
8702	Gyr*H	28	22	23	27	100.
86333	Bon*J	17	30	29	24	100.
8718	Gyr*H	31	23	24	26	104.
86369	Ps*Hr.C	27	28	27	25	107.
86367	C*Hr.N	23	25	30	31	109.
85321	Ps*Hr.C	25	31	32	23	111.
8668	Gyr*H	29	32	25	33	119.
85379	N*Ps.C	33	33	33	32	131.

a: European breeds: H= Holstein, Hr= Red Holstein, J= Jersey, N= Normande, Ps= Brown Swiss.
Criollo breeds: Bon= Blanco Orejinegro, Sm= San Martinero.
Zebu breeds: C= Brahman, Gyr
The asterisks separate sire and dam breed composition.

b: Used to calculate the Kendall's coefficient of concordance ($W = 0.6838$, $P < 0.001$).

Differences in the responses according to the breed composition of the animals were observed. In each test, values of susceptibility to the tick or counts from field infestations were segregated into two groups; one consisting of the Gyr-Holstein heifers and the other of the crossbred castrated males, and then subjected to analysis of variance.

The pattern of responses changed in time. In T1 the Gyr-Holstein group displayed significantly ($P < 0.05$) higher susceptibility (mean 1.95%) than the crossbred (mean 0.96%). In test two (T2) when the counts of ticks from field infestations were performed, no significant differences in tick numbers were observed between groups. In T3 the differences in susceptibility to the tick were not significant (Gyr-Holstein = 5.14%, crossbred = 7.03%). Finally, in T4 the differences between groups were significant ($P < 0.05$), but this time the Gyr-Holstein animals showed lower susceptibility (mean 0.41%) than the crossbred (mean 1.24%).

9.3.2.- Skin test responses.

Reactions induced by the inoculation of the various materials varied according to the antigen used in each test. The levels of the response also varied on different occasions. Figure 9.3 illustrates the results of the parameter diameter of reaction measured at 30 minutes post injection. In each test, the differences between the reactions to the various antigens used, were significant ($P < 0.05$), but showing different patterns from test to test. Reactions tended to be lower in test two.

Antigens P3 and GSG were used in all the tests. Reactions to P3

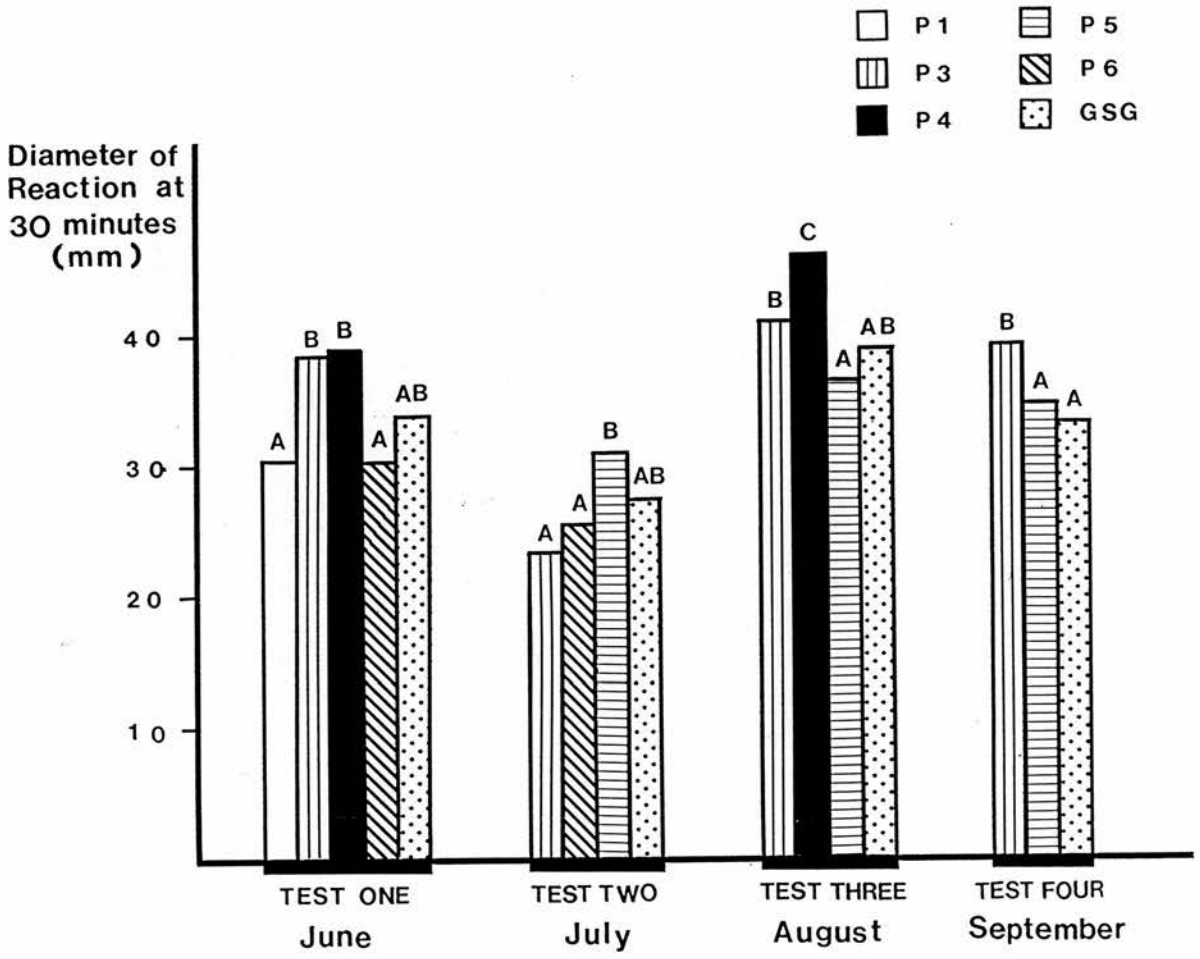


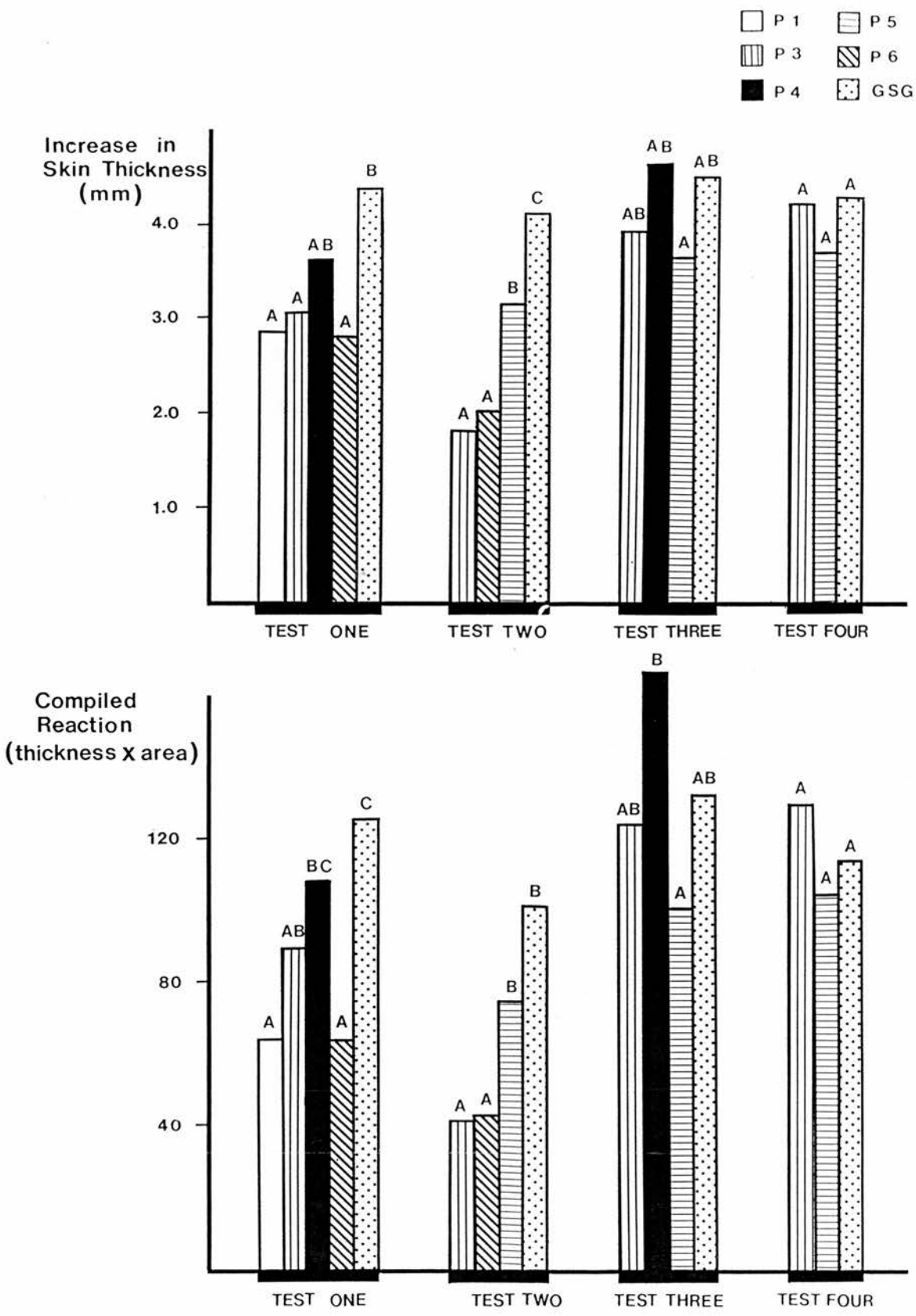
Figure 9.3. Skin hypersensitivity responses to the inoculation of antigens derived from the tick Boophilus microplus in animals immune to the tick and repeatedly tested under field conditions. Means of diameters of the reactions at 30 minutes post inoculation are presented. In each test, bars accompanied by the same letter are not significantly different ($P > 0.05$). P1, P3, P4, P5 and P6 = antigens separated from larval extracts by anion-exchange chromatography GSG = homogenate of semi-engorged female's salivary glands.

at 30 minutes on T2 (mean 23.4) were significantly smaller ($P < 0.05$) than reactions found on T1 (38.7), T3 (40.9) or T4 (38.9) whose levels of reactivity did not differ. Reactions to GSG at 30 minutes showed a similar pattern, the mean value for T2 was 27.5 which was significantly smaller than values found for T1 (34.0) or T4 (33.1). Values found for T3 (38.4) were significantly higher ($P < 0.05$) than reactions found in the other tests.

A comparison of two parameters (increase in skin thickness and compiled reaction = thickness x area) used to measure the reactions at four HPI is presented in figure 9.4. It can be seen that reactions followed a pattern similar to that observed in reactions at 30 minutes. Lower reactivity to the antigens was noticed in test two. Results on both parameters were very similar. Values obtained on the parameter compiled reaction showed higher variances which were not homogeneous between the different antigens, but still the significance of the differences on the responses to different antigens was equally identified in both parameters.

The different parameters used to measure the reactions (diameter at 30 minutes post injection, diameter at 4 HPI, increase in thickness at 4 HPI and compiled reaction at 4 HPI) were then compared by correlation analysis. Using data from all the tests and to all antigens and dilutions used (total observations = 1413). All the measures of the reactions were significantly correlated ($P < 0.001$). The correlation coefficients observed between the reactions at 30 minutes and at 4 hours were: 0.67, 0.57 and 0.59 respectively for diameter, thickness and compiled reaction. However, when values were analysed independently for each antigen, for each dilution and in each test this relationship was not always so strong.

Figure 9.4. Comparison of two parameters used to measure delayed hypersensitivity reactions to the inoculation of Boophilus microplus derived antigens in cattle with various degrees of resistance to the tick, and repeatedly tested. In each test and for each parameter bars bearing not the same latter differ at a significant level ($P < 0.05$). P1, P3, P4, P5 and P6 are antigens purified from larval extracts by anion-exchange chromatography GSG = salivary gland homogenate.


P 1
P 5
P 3
P 6
P 4
GSG
Increase in Skin Thickness (mm)
4.0
3.0
2.0
1.0
A
A
AB
A
B
A
A
B
C
AB
AB
A
AB
A
A
TEST ONE
TEST TWO
TEST THREE
TEST FOUR
Compiled Reaction (thickness x area)
120
80
40
A
AB
BC
A
C
A
A
B
B
AB
B
A
AB
A
A
A
TEST ONE
TEST TWO
TEST THREE
TEST FOUR

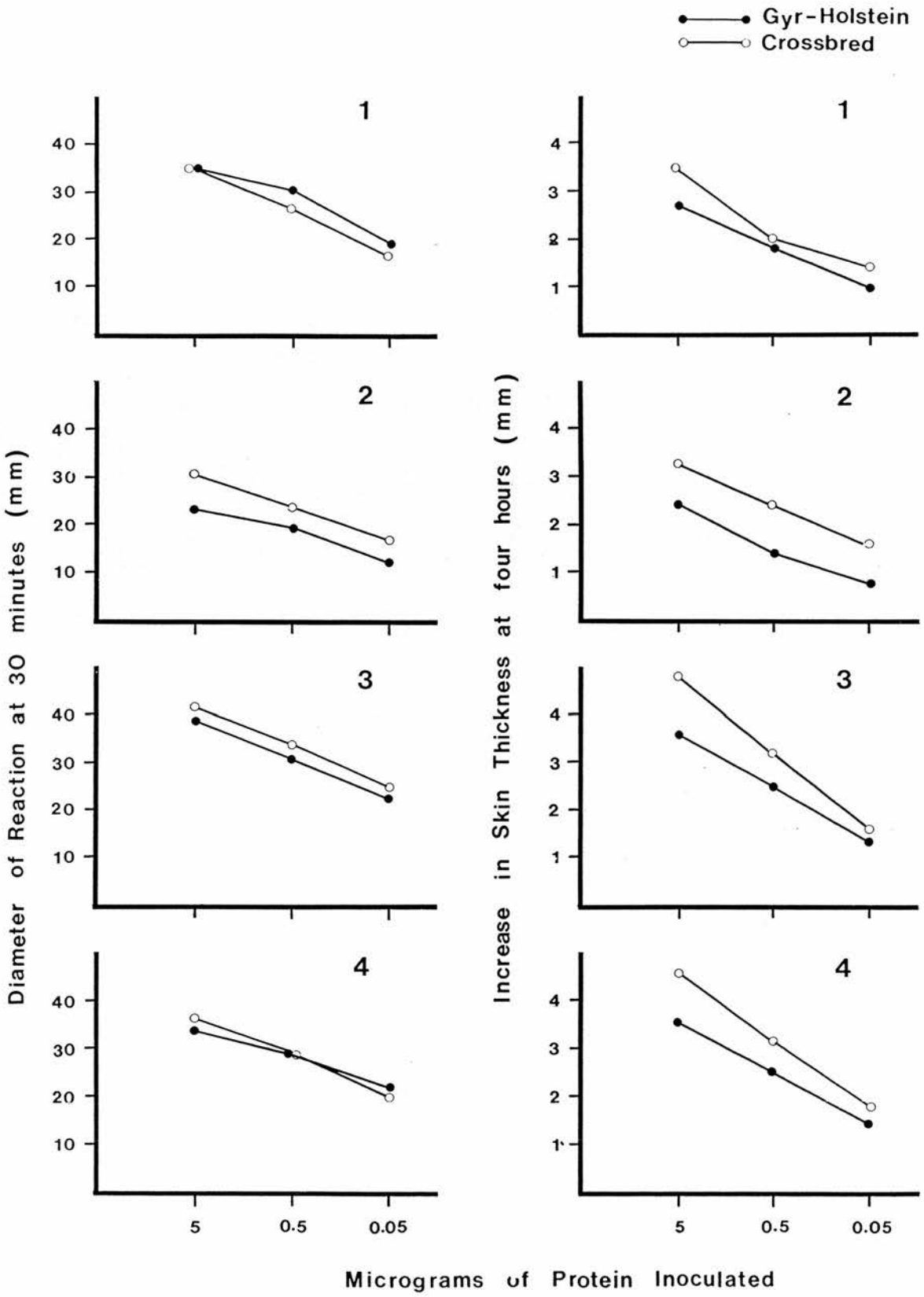
The correlation coefficient between the diameter at 30 minutes and the diameter at 4 HPI ranged from 0.05 to 0.77. The coefficient between the diameter at 30 minutes and the increase in skin thickness at 4 HPI ranged from 0.10 to 0.60 and that between the diameter at 30 minutes and the compiled reaction at 4 HPI ranged from 0.17 to 0.69.

The correlation coefficient between the diameter of the reaction at 4 HPI and the increase in thickness at 4 HPI was 0.68 (n = 1413), it also varied when data were analysed separately ranging from 0.07 to 0.85.

The skin test responses in both groups of animals were then studied using values obtained for all antigens on the parameters diameter at 30 minutes and increase in skin thickness at 4 HPI. Reactions were always stronger in the crossbred group. If the diameter at 30 minutes was used as a measure of the reaction, the differences were significant ($P < 0.05$) in tests two and three. When the increase in skin thickness at 4 HPI was used as a measure of the reaction, the differences between groups were significant in all the tests. A wider separation on the responses between both groups of animals was observed in test two (figure 9.5). The thickness of the normal skin of the animals was also compared. They showed no differences between the tests but the mean skin thickness in the Gyr-Holstein animals (8.5 ± 0.19, mean $\pm$ S.E.) were smaller ($P < 0.001$) than those values observed on the crossbred (10.6 ± 0.13).

9.3.3.- Anti-tick antibodies in the experimental animals and changes in the titres in the various tests.

Figure 9.5. Comparison of the general skin reactivity to the dermal inoculation of antigens derived from the Boophilus microplus in cattle with various degrees of resistance to the parasite, grouped according to their breed composition and tested repeatedly at five week intervals (test one to four). Plotted points are the means of the parameters, diameter of reaction at 30 minutes, and increase in skin thickness at four hours post injection, calculated on either 18 Gyr-Holstein heifers (12 in test one) or on 15 crossbred (various Zebu x European crosses) castrated males (14 on test one). For each animal, the mean value of reactivity to all antigens used on that test and on the corresponding concentration of protein, was used for the analysis. The numbers indicate the test to which values belong.


Gyr-Holstein
Crossbred
1
2
3
4
40
30
20
10
4
3
2
1
Diameter of Reaction at 30 minutes (mm)
Increase in Skin Thickness at four hours (mm)
5
0.5
0.05
Micrograms of Protein Inoculated

ELISA titres in the animals ranged from 2.3 to 9.4 (values are expressed as the base 2 logarithm of the reciprocal of 1/100 of the dilution used). Individual responses of the animals in the various tests were correlated (T1/T2: $r = 0.80$, T1/T3 $r = 0.77$, T2/T3: $r = 0.86$, all: $P < 0.001$).

A two way analysis of variance detected highly significant differences ($P < 0.001$) in the antibody titres according to the test and to the group of cattle evaluated. Mean titres were always higher on the Gyr-Holstein group (5.33, 5.78 and 4.93, respectively for tests one, two and three) than in the crossbred group (3.37, 4.38 and 3.64). The mean values observed in test two were significantly ($P < 0.05$) higher.

9.3.4.- Analysis of the relationship between levels of resistance to the tick, responses in the skin tests and titre of anti-tick antibodies.

To study the relationship between the susceptibility to the tick and the skin test responses a multiple matrix of correlations was prepared as described in previous chapters (5.2.3, 7.3.4). A summary of the significant correlations found is displayed in table 9.3.

The pattern of significant ($P < 0.05$) correlations between the level of susceptibility to the ticks on the animals and the size of the skin responses to each antigen at each dilution, differed in each test. Coefficients for the same antigen using either of the two measures of the reaction (diameter at 30 minutes and increase in thickness at 4 hours) were not equal. No correlations were observed

Table 9.3. Correlation coefficients of the relationship between numbers of ticks Boophilus microplus counted on the animals and the size of the hypersensitivity reactions to the dermal inoculation of tick-derived proteins.

		CORRELATION COEFFICIENTS	
Test +	Antigen @	Diameter at 30 minutes	Increase in Thickness at four hours
T1	P3 - A	0.12	-0.30
	P3 - B	0.41*	-0.27
	P3 - C	0.15	0.01
	GSG - A	-0.19	-0.45*
T2	P3 - A	-0.11	-0.07
	P3 - B	-0.07	-0.22
	P3 - C	-0.22	-0.37*
	P5 - A	-0.37*	0.16
	P5 - B	-0.30	-0.06
	P5 - C	-0.26	-0.45**
	GSG - A	-0.27	-0.28
	GSG - B	-0.20	-0.38*
	GSG - C	-0.23	0.02
T3	P3 - A	-0.24	-0.23
	P3 - B	-0.04	-0.13
	P3 - C	-0.10	-0.07
	P4 - A	0.02	-0.02
	P4 - B	0.02	-0.36*
	P4 - C	-0.07	0.06
	GSG - A	0.06	-0.10
	GSG - B	-0.07	0.10
	GSG - C	-0.08	-0.23
T4	P3 - A	-0.12	-0.10
	P3 - B	-0.28	0.04
	P3 - C	-0.05	-0.16
	GSG - A	0.02	-0.14
	GSG - B	-0.09	-0.20
	GSG - C	-0.06	0.03

\+ : Correlations for T1, T3 and T4 were calculated with the parameter transformed susceptibility to ticks and those for T2 with the parameter log mean count.

@ : Micrograms of protein inoculated into the skin:
- A = 5, - B = 0.5, - C = 0.05

* = Significant ($P < 0.05$) correlation coefficient.

** = Highly significant ($P < 0.01$) correlation coefficient.

in test four.

The reactions displayed by the animals to the different antigens at each dilution and in each test were then ranked separately for reactions at 30 minutes and at 4 HPI and compared to the rank on susceptibility to the tick assigned to the animals in each of the test (9.3.1), using Spearman rank correlation coefficients (Siegel, 1956). The more relevant results are presented in table 9.4.

The pattern of significant correlations displayed by this new arrangement was similar to that seen in table 9.3.

In test one, the reactions at 4 HPI to the antigen P3 at two of the concentrations used, were significantly correlated ($P < 0.05$) with the ranks on susceptibility to the tick. Surprisingly a positive correlation was observed with the reactions at 30 minutes using 0.05 micrograms of protein of this antigen. The ranks on the reactions to GSG at 4 HPI in the unique dilution used in this test also were correlated negatively with the ranks on susceptibility to the tick.

In test two, no correlations were found with reactions to antigens P3 or P6. Negative, highly significant ($P < 0.01$) correlations were observed with reactions at 4 HPI to antigens P5 (only in the smallest concentration used) and GSG. No correlations were observed in tests three or four.

The ranking of the reaction in the skin test allowed non-parametric comparisons between the reactions produced by each antigen at the three concentrations studied in each test, using for the comparison both the measures at 30 minutes and at 4 HPI. A Kendall coefficient of concordance was calculated for the six

Table 9.4. Spearman rank correlation coefficients obtained by comparing the ranks on counts of Boophilus microplus engorged females to the ranks on the dermal responses to the inoculation of tick-derived antigens in animals previously exposed to the tick. Dermal responses were measured at 30 minutes (diameter of reaction) or at 4 hours post injection (increase in thickness).

		SPEARMAN CORRELATION COEFFICIENT (r')	
Test	Antigen @	Reaction at 30 minutes	Reaction at 4 hours
T1	P3 - A	0.01	-0.40*
	P3 - B	0.33*	-0.41*
	P3 - C	-0.02	-0.29
	GSG - A	-0.20	-0.49*
T2	P3 - A	-0.21	-0.26
	P3 - B	0.005	-0.15
	P3 - C	-0.23	-0.21
	P5 - A	-0.29	-0.17
	P5 - B	-0.19	-0.14
	P5 - C	-0.21	-0.44**
	GSG - A	-0.27	-0.38*
	GSG - B	-0.14	-0.48**
	GSG - C	-0.24	-0.10
T3	P3 - A	-0.15	-0.27
	P3 - B	-0.03	-0.10
	P3 - C	-0.10	0.20
	GSG - A	0.12	-0.10
	GSG - B	-0.12	0.05
	GSG - C	-0.19	-0.17
T4	P3 - A	-0.07	0.07
	P3 - B	0.16	0.24
	P3 - C	0.08	0.08
	GSG - A	0.04	-0.11
	GSG - B	0.09	-.015
	GSG - C	0.05	-0.22

@ : Micrograms of protein inoculated on the skin:
- A = 5.0, - B = 0.5, - C = 0.05

* = Significant ($P < 0.05$), Spearman correlation coefficient.

** = Highly significant ($P < 0.001$), Spearman correlation coefficient.

measures of reactivity available for each antigen (three concentrations, and on each concentration the diameter of the reaction at 30 minutes and the increase in thickness at four HPI). Results are displayed in table 9.5. For all the antigens and in the four tests, the ranks on the reactivity to the different concentrations of the materials under study using any of the two measures of the reaction, were very similar as indicated by a highly significant Kendall coefficient of concordance.

Animals were also ranked on their anti-tick antibody titre. This rank was used to calculate Spearman rank correlations with the ranks on the tick count in the corresponding test. Correlations were also calculated with the rank on the general reactivity to each of the antigens in each test. Results are displayed in table 9.6.

No significant correlations were found between the tick count and the anti-tick antibody titre. However, the higher coefficient was observed in test two ($r' = 0.28$, $P < 0.10 > 0.05$). The general reactivity to the antigen P3 was negatively correlated with antibody titres also in test two ($r' = -0.43$, $P < 0.05$). The general reactivity to the antigen GSG was negatively correlated with the antibody titre in tests two and three (respectively, $r' = -0.42$ and -0.43, both $P < 0.05$).

The Spearman rank correlation coefficients between tick count and general reactivity to the antigens P3 and GSG are also presented in table 9.6 for comparative purposes. Note that both the reactivity to P3 or to GSG were negatively correlated with the tick count in test two (respectively, $r' = -0.34$ and -0.37, both $P < 0.05$). In test one the general reactivity to GSG was negatively correlated with the tick count ($r' = -0.41$, $P < 0.05$).

Table 9.5. Kendall coefficient of concordance (W) calculated on the dermal reactions displayed by 33 cattle immune to Boophilus microplus ticks, to the inoculation of tick-derived antigens. For each antigen in each test two measures of the reactions (diameter at 30 minutes, increase in skin thickness at four hours) were used in each of the three concentrations studied (6 values for antigen).

Test	Antigen	W	chi square	P
T1	P1	0.528	79.18	< 0.001
	P3	0.471	70.62	< 0.001
	P4	0.444	60.66	< 0.001
	P6	0.546	81.98	< 0.001
	GSG+	0.710	35.50	0.10 > P > 0.05
T2	P3	0.407	78.10	< 0.001
	P6	0.382	73.44	< 0.001
	P5	0.634	121.78	< 0.001
	GSG	0.495	94.96	< 0.001
T3	P3	0.482	92.57	< 0.001
	P4	0.548	105.33	< 0.001
	P5	0.414	79.51	< 0.001
	GSG	0.578	110.91	< 0.001
T4	P3	0.521	100.08	< 0.001
	P5	0.356	68.45	< 0.01
	GSG	0.375	72.06	< 0.001

+ = Only one concentration of antigen studied.

Table 9.6. Relationship between the ranks on the counts of Boophilus microplus ticks, the general reactivity to the inoculation of the tick-derived antigens P3 or GSG, and the anti-tick antibody titre in 33 cattle immune to the tick and repeatedly tested. La Libertad research centre. Villavicencio. Colombia.

	SPEARMAN CORRELATIONS WITH ANTIBODY TITRE ON EACH TEST		
	T1	T2	T3
Tick count	0.07	-0.28	-0.06
P3 reactions	0.11	-0.43*	-0.19
GSG reactions	-0.24	-0.42*	-0.34*

	SPEARMAN CORRELATIONS WITH TICK COUNT ON EACH TEST			
	T1	T2	T3	T4
P3 reactions	-0.19	-0.34*	-0.07	0.13
GSG reactions	-0.41*	-0.37*	-0.08	0.04

* = Significant coefficient ($P < 0.05$).

9.3.5.- Meteorological records.

For a comprehensive illustration, the meteorological records of 1987 and 1988 are presented. The changes in temperature, relative humidity and rainfall are displayed in figure 9.6.

In the region of study, the temperature remains relatively unchanged throughout the year, fluctuating from 20°C to 35°C. Higher temperatures are recorded during the dry season (December-March). The relative humidity decreases drastically (under 70%) during the dry season, particularly towards the end (March), but it returns rapidly to levels over 70% after the first few rains. 1987 had a milder dry season than 1988.

9.4.- DISCUSSION.

The most remarkable finding during this experiment was the variability in the responses in the susceptibility to the tick, in the reactivity in the skin tests and in the antibody titre to salivary gland antigens, as they were repeatedly evaluated using the same animals. It was also noticeable that the degree to which those measures were interrelated changed in the different evaluations. The discussion will focus on the explanation of the factors related with that variability.

The susceptibility to the tick was significantly higher when measured in August (T3), which seems to be related to the higher contamination of the pastures with tick larvae. However, peak levels of tick infestation have been described towards the middle of the rainy season (August) in studies conducted on animals grazing

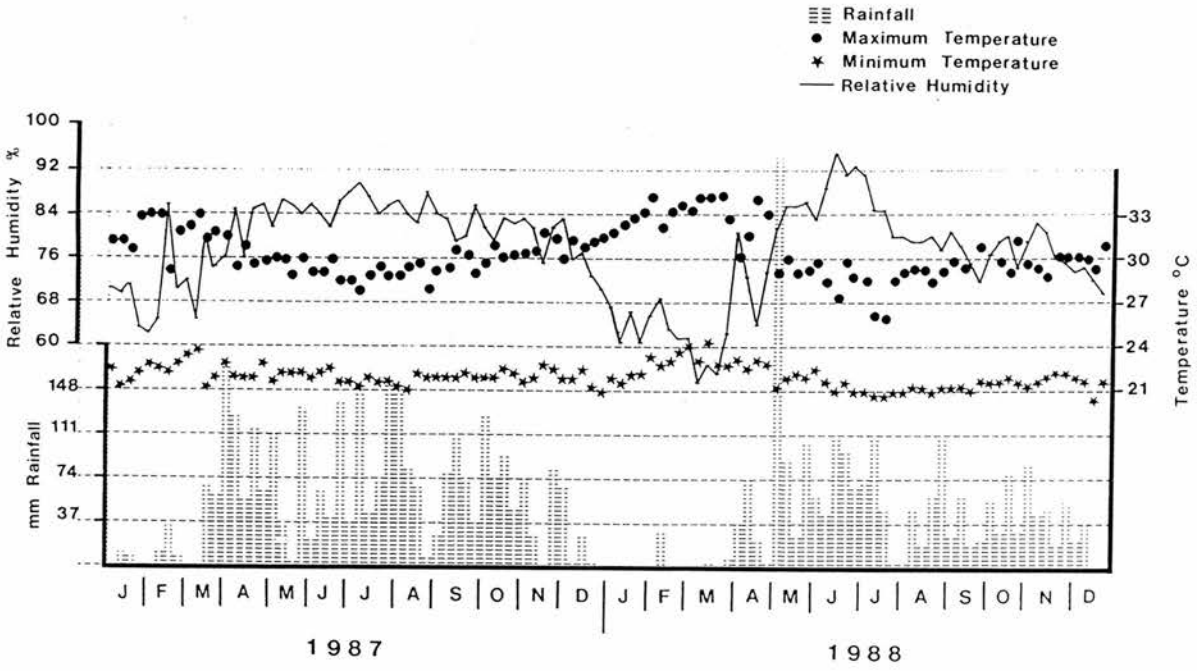


Figure 9.6. Weekly fluctuation in minimum and maximum temperature, relative humidity and rainfall at La Libertad research centre, Villavicencio, Colombia. Rainfall values are the addition of daily observations. In the other parameters the presented value is the mean value of the observations of that particular week. Records of 1987 and 1988 are presented.

Brachiaria decumbens on the well drained savannas of the eastern plains of Colombia (Aycardi et al., 1984) and on the foothills of those savannas (Benavides, Villar and Gonzalez, 1988), not necessarily related with high counts of larvae in the pastures.

If the higher susceptibility to the tick observed in August was due to reductions in the resistance status of the animals, related to nutritional or enviromental factors such as those described by Sutherst et al. (1983a), or if it was only caused by the higher larval contamination of the pastures, is not clear.

Although the tick counts in the different tests were significantly correlated, lower coefficients were observed between T1 and T4 and between T2 and T4. This could indicate that some animals changed their status of resistance. This finding agrees with those of Madalena et al. (1985), who reported low correlation in tick counts performed in different seasons on the same animals, and that too low or too high tick burdens decreased the correlations.

A closer examination of table 9.2 showed that 13 out of 18 of the Gyr-Holstein heifers decreased on their rank when values in T1 and in T4 were compared. When the results in the percentages of susceptibility obtained by each animal in T1 and T4 were weighed, it was seen that 17 out of the 18 Gyr-Holstein heifers had a lower susceptibility in T4 than in T1, whilst 9 of the 15 crossbred males had higher susceptibility in T4 than in T1 (chi squared = 9.049, $P < 0.01$). In the crossbred group, 4 animals were equally ranked in both tests.

On the basis of these results, it could be thought that the Gyr-Holstein heifers had not attained a stable level of resistance at the beginning of the experiment. The crossbred group of males

displayed less variability in their susceptibility to the tick. On the other hand, although all animals showed higher tick counts resulting from the artificial infestation in T3, the counts in T3 and T4 were closely correlated. That is probably a reflection of a more stable level of resistance being attained in both groups.

With reference to the reactivity displayed for the animals in the skin test, significantly lower levels of reactivity were observed in T2, which appears to be a reflection of an immunosuppressive factor acting on the animals at that time. Tick numbers were also high then and it is not clear if both phenomena were caused by an environmental or managerial factor (nutrition), or if the high tick numbers were causing the observed immunosuppression through their anorectic and specific effects (O'Kelly, Seebeck and Springell, 1971). T2 was conducted in July, which corresponds to the middle of the rainy season, the protein and mineral content of Brachiaria decumbens have been stated to decrease at this time of the year (Laredo and Gomez, 1980).

Anti-tick immunoglobulin G (IgG) titres, as measured by the ELISA test were significantly higher in test two in both groups of animals, when a weak positive correlation with tick counts was found. In all the three tests evaluated, the titres were significantly higher on the Gyr-Holstein group.

There is no simple explanation for these results. Brossard, (1976) using an indirect immunofluorescence test, demonstrated peak antibody titres to salivary gland antigens of Boophilus microplus after the initial infestation of cattle with ticks, declining thereafter over a period of months once the infestation was

finished, and in chapter seven (7.3.3) it was demonstrated that the antibody titres increase steadily after the sequential exposure of the calves to tick. However, in the literature searched, no report was found on the long term evolution of anti-tick antibody titres in animals permanently infested with ticks.

The results obtained here suggest that anti-tick antibody titres in cattle permanently infested with ticks are of a lower level than those observed in cattle recently exposed. This is emphasized by the fact that higher antibody titres were found at the time of maximum tick infestation, in some way contradicting the theory of immunosuppression. However it is probable that high antibody titres are only developed in animals that allow high numbers of ticks to feed on them, not having a direct relationship with the expression of resistance to the tick.

This may be the cause of the weak positive correlation with the susceptibility to the tick (higher antibody titre to increased susceptibility) found in this experiment, as opposed to the findings of chapter seven (7.3.4), and agrees with the findings of Willadsen et al., (1978), who used a purified antigen from B. microplus larvae in an indirect haemagglutination test, and described a negative association between resistance to the tick and antibody titres.

Finally, the reactivity in the skin test for antigens P3 and GSG was negatively correlated with both the susceptibility to the tick and the anti-tick antibody titre, but those relationships were only significant in tests one and two. These results indicate that the high levels of IgG antibodies interfered with the immediate hypersensitivity response to the tick-derived antigens, which is a normal mechanism exploited in hyposensitization therapies (Roitt,

Brostoff and Male, 1985). This explains the fact that the stronger correlations were found at the time that the higher antibody titres were recorded.

A similar and related phenomena appears to occur with the measure of the relationship between the reactivity in the skin test and the susceptibility to the tick. It seems that under heavy tick challenge, the more susceptible animals lose their capacity to mount a hypersensitive response to the inoculation of tick-derived antigens, as strong as that displayed when they are not under such a challenge. It is not possible to elucidate from the results of this experiment if this same phenomenon occurs by the inoculation of any non tick-derived antigen.

9.5.- CONCLUSIONS.

- Changes in the level of susceptibility to the tick B. microplus, as measured by artificial infestations with 20,000 larvae were displayed by the experimental animals. Higher levels of susceptibility were observed in August. Higher levels of contamination of the pastures with larvae and high tick burdens on the animals were observed in July; the corresponded to the middle of the rainy season in the area of study.
- Although all animals were similarly ranked for tick counts in all the evaluations of this study, slight differences were observed on the consistency of the ranks between the Gyr-Holstein heifers and the crossbred males indicating that the former group had not acquired a solid resistance to the parasite at the begining of the experiment. A more stable level

of resistance was achieved by both groups in the last evaluations.

- Lower levels of reactivity in the skin test and higher antibody titres to salivary gland antigens were observed in T2 conducted in July, when correlations were observed between those responses and the tick count on the animals. It was suggested that high titres of IgG antibodies, which were developed only in animals experiencing heavy tick challenge, interfered with the hypersensitivity reactions to tick-derived antigens, which would explain why correlations between the tick counts, the skin test reactivity and the antibody titres to salivary gland antigens were only observed in the times of heavy tick challenge.

CHAPTER TEN:

GENERAL DISCUSSION

The majority of objectives proposed at the beginning of this study were fulfilled. Using chromatographic techniques, various antigens for use in the skin test were purified from larval extracts of Boophilus microplus. Skin tests using these materials were compared under field conditions with the standard methods used to measure tick resistance in cattle. The feasibility and the limitations for the implementation of these techniques in a medium sized laboratory in Colombia, and for their application in cattle maintained under tropical conditions were studied.

Results for each separate experiment were discussed in detail in each chapter, thus the general connexion between the different experiments, and the findings relevant to the general objectives of this work will be discussed here before arriving at conclusions about the probable use of the skin test for the selection of cattle under tropical conditions.

A good degree of separation of proteins from larval extracts of either R. appendiculatus or B. microplus ticks was obtained using anion-exchange chromatography, as demonstrated by the SDS-PAGE profiles of the different materials obtained for each tick species. For various reasons, including the lack of proper reagents, SDS-PAGE was not conducted at LIMV. Limited quantities of semi-purified proteins were freeze dried and transported to CTVM. Thus, a limited amount of material was available for analysis by SDS-PAGE. In addition different anion-exchangers were used in both laboratories.

This fact restricted the comparison of the materials separated from B. microplus larval extracts at both locations. For these reasons they were given a different nomenclature (PK1, PK2, PK3 and PK4, for those produced at CTVM; and P1, P3, P4, P5, P6 and D2, for those produced at LIMV) and were studied in separated experiments. Comparison between materials obtained from both tick species, to explore the existance of cross reactivity or the presence of common antigens, was not attempted, since this was out of the scope of this study.

The laboratory model using R. appendiculatus ticks and rabbits, proved useful for the preliminary standardization of skin test methods and procedures to manipulate the data. However, the skin reactions in immune rabbits were different from those observed on cattle resistant to B. microplus. Reactions in rabbits were characteristic of delayed type hypersensitivity, whilst those seen in cattle were of immediate type hypersensitivity.

The rabbits readily acquired resistance to repeated infestations with R. appendiculatus ticks. A negative correlation between the skin test responses to GSG (from R. appendiculatus) at 24 HPI with the susceptibility to the tick was observed. A negative faint correlation was also found between antibody titres to GSG and susceptibility to the tick. Sera from immune rabbits recognized five antigenic bands (molecular weights: 19, 33, 34, 48 and 52 kD) in GSG. Of these the 33 and 34 kD bands, appear to be the 36-38 kD bands described by Walker, Fletcher and Todd (1989) in salivary homogenates from different instars of R. appendiculatus.

The results of the experiments using B. microplus and cattle were more difficult to evaluate, for various reasons. To start with,

environmental factors exerted a strong influence on the experimental animals and hence on the results. Animals used for the evaluations were not always of the same breed composition and were subjected to different management procedures in each experiment, and as explained above, materials prepared at CTVM or at LIMV were considered as separate entities.

A preliminary evaluation of the materials fractionated from B. microplus larvae at CTVM (chapter six), showed that cattle previously exposed to B. microplus, developed immediate hypersensitivity reactions to the inoculation of all the materials tested. However, at four hours, reactivity was also evident for most of the materials evaluated, particularly for the larval derived materials PK1 and PK3 when tested on Criollo cattle. No reactivity was observed at 24 HPI.

In the subsequent experiment (chapter seven), the evolution of the responses on naive calves up to the fourth infestation with ticks was studied. It was demonstrated that the responses in the skin test were immune specific. The reactivity in the skin test increased markedly as the animals were exposed to the tick, despite the fact that a slight inflammatory reaction was observed in tick-naive animals, particularly for GSG. No previous reports were found on the evolution of the skin test reactivity to B. microplus antigens in cattle repeatedly exposed to this tick. Antibody titres to GSG were raised in accordance with the repeated exposure of the calves to the tick, this has been discussed already (7.4).

The evaluation of the materials purified from B. microplus larvae at LIMV, followed a similar strategy. The different materials

were initially tested on animals previously exposed to the tick (chapter eight) and then were assayed repeatedly under more controlled conditions (chapter nine). These materials showed similar skin test reactivity to that produced for materials purified at CTVM. Thus, the reading of reactions at 24 hours was not attempted in the final experiment.

The repeated evaluation of 33 adult cattle, maintained in a paddock in the tropics (chapter nine), demonstrated that the skin test reactivity, the susceptibility to the tick and the antibody titres to GSG fluctuated with time. Lower levels of reactivity in the skin test occurred contemporarily with the highest tick burdens and with the highest antibody titres. Although this variability was partially attributed to the fact that some animals had not acquired a solid resistance to the tick, the evidence strongly suggests that there was a marked seasonal effect on all the responses as described for the resistance to the tick in Australia (Sutherst et al, 1983b). Confirmation of this would require a longer study.

In all the experiments described above, associations of different strength were observed between the responses in the skin test and the level of susceptibility to the tick on the animals, and between both of them with the titre of antibodies to GSG. The strength of these associations appears to be influenced by many factors, some related to environmental constituents and others related to the distribution of the responses in the particular group of animals being evaluated.

Significant negative correlations between susceptibility to the tick and the skin test responses to antigens PK1, PK3 and GSG were observed when 21 Holstein and 5 crossbred cattle were tested

together, but when 12 of the Holstein were re-evaluated a few months later, no correlation was found between these responses. At this time, skin test responses were stronger and the animals were less susceptible to the tick than in the first test (chapter six).

In the subsequent experiment, when a group of Gyr-Holstein calves were repeatedly evaluated from naive up to the fourth infestation under controlled conditions, that relationship was found to be significant for antigens PK1, PK2, PK3 and GSG (both 30 minutes and 4 HPI readings). On the same experiment a weak negative correlation was found between antibody titres to GSG and the susceptibility to the tick, and those antibody titres were positively correlated with the skin reactivity to all the antigens.

It should be noted that in this experiment, the animals were kept on a high nutritional plane and stalled on the Tibaitatá research centre, where there is no effect of heat stress or other stress factors commonly found in the tropics. At the same time, the arrangement of the experiment influenced the observed degree of correlation between the variables, because naive animals are more susceptible to the tick and at the same time show weaker skin reactions and lower antibody titres to GSG than tick exposed animals. In this form the skin test and the determination of antibodies by ELISA were useful to discriminate between animals exposed or unexposed to the tick, but it was not clear if these tests were able to discriminate animals of different levels of resistance to the tick. This led to the subsequent experiments, conducted in animals which had acquired stable levels of resistance and were maintained under tropical conditions.

In the ensuing experiment (chapter eight) when different groups of animals were evaluated there was remarkable variability in the degrees of association found between the skin test responses and the level of susceptibility to the tick, in spite of the introduction of non-parametric statistics to reduce the influence of the distribution of the responses on the measure of the relationships. The correlations between skin test responses and the susceptibility to the tick varied according to the following factors: antigen used, time for reading the reaction after injection, group of animals evaluated, and time of the year in which the test was performed.

In the final experiment of this study, when a group of 33 crossbred cattle were repeatedly evaluated at five week intervals, it was observed that correlations between tick burden and skin test responses, and between skin test responses and anti-tick antibody titres, were only evident in July, at a time when the animals were subjected to heavy tick burdens. The significance of these findings was already discussed in the relevant chapter, but is interesting to note that in Australia, Stear et al. (1989), found significant associations between major histocompatibility type and tick resistance only in July, when the mean and variances of ticks numbers were the lowest.

In this experiment the opposite ocurred. During July the variances of the skin test responses were the highest. It appears that although in general, animals were similary ranked for susceptibility to the tick on all the evaluations, it was only in July, that the tick burdens carried for the animals exerted a deleterious effect on the skin responsiveness of the animals to the tick derived antigens. That decrease in skin test responsiveness in

animals carrying heavy tick burdens, might be the cause of the observed associations.

It could be argued that an external factor (for example nutrition) could cause the loss of resistance to the tick in the animals and the low responsiveness in the skin test, a fact that cannot be discounted. This appears to have occurred during the second experiment in chapter six, when no correlation was seen between skin test responses and tick burdens. There, when the nutrition and the management of the animals were improved, they displayed stronger skin test responses and were less susceptible to the tick. Both ticks and inappropriate nutrition are stress factors commonly found in the tropics, and the immune response is subjected to their influences (Frisch, 1981).

Two final questions arise from this study. Is the skin test useful for the selection of animals on the basis of their capacity to display resistance to the tick and if so, which is the more relevant antigen to be used?.

The answer to the first question is yes, but probably with a different emphasis as initially thought. The skin test responses were demonstrated to be affected by stress factors such as poor nutrition or tick infestation. Thus, in the context of its use as a tool for the selection of animals on the basis of their capacity to acquire strong resistance to the tick, the only advantage displayed for the skin test over the method of ranking the cattle on the basis of tick counts, is that the animals could be alleviated earlier from tick infestation by treatment with acaricide and the animals could still be ranked for resistance to the tick on the basis of their

skin test reactivity.

Marked differences in the skin responses between animals of high and low resistance were observed in times of heavy tick challenge, and susceptible animals displayed stronger skin reactions when the stress stimuli had disappeared. This means that in the absence of stress the differences in skin test reactivity between both groups of animals would be minimal, making it difficult to discriminate between them. Alternatively, it was suggested that in animals displaying resistance to the tick, increases in titres of IgG anti-tick antibodies interfered with the skin test reactivity. Such increases in antibody titre were observed at times of heavy tick burdens, and that interference would partially explain the low skin test reactivity observed at such times.

Returning to the potential use of the skin test for selecting animals on the basis of resistance to the tick. It could be used in conjunction with the ELISA test, to detect those animals capable of producing high antibody (IgG) titres in response to tick infestation, and to detect those animals highly responsive in the skin test under all types of field conditions. For this it is important to consider the probability that the selection of animals for resistance to ticks on the basis of both the skin test and the measurement of antibodies by ELISA would require a discriminant (between negatives and positives for example) rather than an associative measure of the relationship. To assess that level of discrimination, further experiments would be required, including repeated evaluations on larger groups of animals, grazing naturally in a tick infested area.

Both GSG and P3 would be the materials of choice to conduct

that type of skin test, but improvements on the purification and characterization of these materials are required. Methods to preserve and to inoculate these antigens under practical conditions in the field should also be ameliorated. The use of freeze dried materials to be dissolved few minutes before use, and the use of a type of prick test (Pepys, 1975), instead of the inoculation of minute volumes of solutions, are recommended for consideration in future experiments.

On the other hand, the skin test could be used as a measure of the adaptability of a particular animal to a stressful environment. Animals maintained in the tropics, and assessed in periods of adverse environmental conditions, could be classified as of low adaptability, when they show faint responses in a standardized skin test. Nowadays it is accepted that IgE and immediate hypersensitivity reactions play an important role in the protection to parasitic worm infections (Roitt, Brostoff and Male, 1985) and ticks (Willadsen, 1980a; Wikel and Whelen, 1986). Thus, cattle selected for hypersensitivity responses would be selected on the basis of their capacity to display this protective mechanism in periods of adverse environmental conditions, and indirectly would be selected for adaptability. To perform this type of selection, any antigen inducing immediate hypersensitivity reactions could be used, even without the necessity of being originated from ticks.

It could be argued that these allergic conditions are detrimental to cattle, and that this type of discrimination will select for an atopic state in cattle. However, allergic dermatitis due to hypersensitivity to tick or insect bites has not been

described on tick resistant animals, and resistant cattle in the tropics appear to maintain their productive and reproductive status unaltered.

Finally, in the experiment at Tibaitatá, where a group of calves were examined from naive to the tick up to the fourth infestation, it was suggested that both the skin test and the ELISA could be used as predictors of the degree of resistance that the animals would develop after exposure to the tick. A negative correlation between the size of reactions to GSG in the naive animals and the level of susceptibility attained after three infestations with the tick was demonstrated. A weak positive correlation between the titre of antibodies developed for the animals after the preliminary infestation with ticks, and the level of susceptibility observed after three infestations was also demonstrated. These facts were discussed in the appropriate chapter, and further research was suggested. Unfortunately, this finding arose during the final analysis of the data, when changes in the design of the experiments was not possible.

However, these predictors could constitute an ideal test for selection purposes. Tick-naive animals could be skin tested using tick-derived antigens (such as GSG) and discriminated into high or low responders. Those high responders would be the animals more likely to develop high resistance to the tick after natural exposure. Alternatively cattle could be tested for anti-tick antibodies by ELISA after a primary infestation with ticks (for example after being moved from the tick free area), and discriminated into those of high or low titres. Those producing the lowest titres would be the most likely to develop high resistance to

the tick on subsequent tick challenge. Further experiments to explore this relationship, testing groups of tick naive animals and then measuring the level of resistance acquired after natural exposure to ticks, are strongly recommended.

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APPENDIX

Raw information for the different experiments

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DATASET : CTVHEB. Chapter five

CASE No.	ANIMAL	ANTIGEN	OEDEMA 30min	ERYTHEMA 4h	ERYTHEMA 24h	ERYTHEMA 48h	INC.THICK-NESS 4h	INC.THICK-NESS 24h	INC.THICK-NESS 48h	GROUP	ANTIBODIES ELISA GSG
1	1A	GSG	205.	485.	2151.	1.	2.91	5.31	1.71	1	4.4
2	1B	GSG	161.	541.	721.	1.	2.01	5.01	1.21	1	3.2
3	1C	GSG	157.	421.	2113.	1928.	1.81	4.41	2.81	1	4.8
4	2A	GSG	157.	463.	1074.	961.	2.81	3.41	0.51	2	3.1
5	2B	GSG	183.	461.	1761.	2521.	2.11	4.11	1.51	2	3.4
6	2C	GSG	157.	273.	457.	361.	0.71	1.31	0.61	2	3.4
7	3A	GSG	196.	547.	2133.	2201.	5.41	6.41	2.91	3	4.5
8	3B	GSG	170.	381.	1441.	1.	2.21	4.31	1.51	3	4.4
9	3C	GSG	256.	622.	2461.	1.	4.01	5.51	1.41	3	3.4
10	4A	GSG	91.	484.	2227.	1.	1.91	2.21	1.01	4	2.3
11	4B	GSG	121.	381.	2022.	2194.	1.91	7.91	4.61	4	3.9
12	4C	GSG	157.	401.	1825.	118.	0.81	4.31	2.41	4	4.0
13	Co1	GSG	169.		714.	1.		1.61	-0.09	5	
14	Co2	GSG	211.	362.	1513.	1486.	1.41	4.51	2.41	5	2.6
15	Co3	GSG	100.	324.	1.	1.	1.71	1.31	0.11	5	3.1
16	Co4	GSG	133.	341.	1.	1.	0.71	0.71	0.61	5	2.4
17	Co5	GSG	121.	211.	1.	1.	1.01	0.41	0.01	5	
18	1A	PK1	91.	144.	463.	885.	0.71	5.11	5.01	1	4.4
19	1B	PK1	1.	1.	526.	307.	0.31	4.81	5.01	1	3.2
20	1C	PK1	170.	145.	273.	341.	0.51	3.31	5.11	1	4.8
21	2A	PK1	57.	65.	381.	869.	0.81	3.51	6.31	2	3.1
22	2B	PK1	73.	145.	290.	526.	0.61	3.31	3.51	2	3.4
23	2C	PK1	82.	379.	651.	419.	2.21	2.71	3.91	2	3.4
24	3A	PK1	131.	211.	551.	1486.	0.81	4.41	4.51	3	4.5
25	3B	PK1	1.	122.	576.	521.	1.11	6.51	7.61	3	4.4
26	3C	PK1	89.	145.	415.	361.	0.81	4.11	5.41	3	3.4
27	4A	PK1	133.	155.	341.	568.	1.41	4.11	4.81	4	2.3
28	4B	PK1	101.	170.	379.	769.	0.41	2.91	5.81	4	3.9
29	4C	PK1	144.	169.	381.	714.	-0.09	4.71	5.01	4	4.0
30	Co1	PK1	111.		681.	507.		5.41	6.51	5	
31	Co2	PK1	1.	211.	703.	484.	1.01	4.81	4.91	5	2.6
32	Co3	PK1	65.	73.	211.	381.	0.81	3.61	3.91	5	3.1
33	Co4	PK1	157.	97.	400.	441.	0.81	4.21	4.41	5	2.4
34	Co5	PK1	82.	144.	290.	576.	0.81	3.01	3.91	5	
35	1A	PK2	82.	273.	649.	1296.	0.91	5.81	6.41	1	4.4
36	1B	PK2	121.	1.	769.	463.	1.01	5.61	6.91	1	3.2
37	1C	PK2	101.	256.	573.	463.	1.81	4.41	7.11	1	4.8
38	2A	PK2	133.	144.	400.	865.	0.51	3.91	5.11	2	3.1
39	2B	PK2	111.	183.	781.	865.	0.31	2.81	5.71	2	3.4
40	2C	PK2	122.	341.	507.	595.	2.21	4.41	6.61	2	3.4
41	3A	PK2	121.	235.	1103.	2366.	2.01	8.71	9.91	3	4.5
42	3B	PK2	100.	244.	461.	1185.	0.91	5.31	8.51	3	4.4
43	3C	PK2	91.	169.	601.	784.	0.81	6.21	7.41	3	3.4
44	4A	PK2	100.	341.	1065.	783.	1.31	4.71	5.21	4	2.3
45	4B	PK2	157.	529.	729.	1703.	1.71	4.31	7.61	4	3.9
46	4C	PK2	111.	289.	841.	1678.	1.01	5.61	6.11	4	4.0
47	Co1	PK2	91.		622.	645.		4.01	6.81	5	
48	Co2	PK2	141.	381.	900.	1089.	1.71	5.41	6.01	5	2.6
49	Co3	PK2	133.	267.	507.	784.	1.41	3.51	3.21	5	3.1
50	Co4	PK2	121.	100.	813.	589.	0.71	4.41	5.91	5	2.4
51	Co5	PK2	101.	225.	625.	900.	1.61	6.41	6.71	5	

CTVMEB.Page2

CASE No.	ANIMAL	ANTIGEN	OEDEMA 30min	ERYTHEMA 4h	ERYTHEMA 24h	ERYTHEMA 48h	INC.THICK-NESS 4h	INC.THICK-NESS 24h	INC.THICK-NESS 48h	GROUP	ANTIBODIES ELISA GSG
52	1A	PK3	131.	100.	144.	1.	0.91	0.81	0.51	1	4.4
53	1B	PK3	1.	183.	1.	1.	0.71	1.11	0.21	1	3.2
54	1C	PK3	145.	169.	1.	1.	0.01	-0.39	-0.19	1	4.8
55	2A	PK3	89.	145.	1.	1.	0.41	0.61	-0.19	2	3.1
56	2B	PK3	155.	145.	211.	111.	0.01	0.71	-0.29	2	3.4
57	2C	PK3	122.	241.	241.	183.	0.71	0.81	0.31	2	3.4
58	3A	PK3	145.	241.	381.	100.	1.11	0.91	0.11	3	4.5
59	3B	PK3	101.	111.	91.	61.	0.61	0.11	0.11	3	4.4
60	3C	PK3	1.	145.	121.	1.	0.11	0.81	-0.19	3	3.4
61	4A	PK3	100.	144.	1.	1.	0.81	0.31	-0.19	4	2.3
62	4B	PK3	145.	337.	256.	1.	0.81	0.21	-0.19	4	3.9
63	4C	PK3	121.	155.	1.	1.	0.11	-0.19	0.41	4	4.0
64	Co1	PK3	57.		1.	1.		0.81	0.51	5	
65	Co2	PK3	111.	421.	1321.	1621.	1.11	3.81	1.71	5	2.6
66	Co3	PK3	111.	144.	64.	1.	0.91	0.61	0.31	5	3.1
67	Co4	PK3	111.	121.	1.	1.	0.31	0.71	0.11	5	2.4
68	Co5	PK3	50.	122.	1.	1.	0.41	0.81	-0.19	5	
69	1A	PK4	144.	256.	441.	1.	1.01	2.01	0.51	1	4.4
70	1B	PK4	1.	1.	1.	1.	0.61	0.21	0.21	1	3.2
71	1C	PK4	141.	273.	461.	1.		1.21	-0.19	1	4.8
72	2A	PK4	122.	211.	91.	1.	0.41	0.21	-0.19	2	3.1
73	2B	PK4	145.	183.	401.	131.	0.51	1.61	-0.29	2	3.4
74	2C	PK4	1.	157.	271.	1.	0.81	0.91	0.31	2	3.4
75	3A	PK4	181.	271.	441.	529.	1.81	2.51	0.11	3	4.5
76	3B	PK4	100.	111.	109.	1.	0.21	0.11	0.11	3	4.4
77	3C	PK4	101.	133.	157.	1.	0.71	1.51	-0.19	3	3.4
78	4A	PK4	1.	122.	1.	1.	0.11	0.21	-0.19	4	2.3
79	4B	PK4	121.	271.	441.	1.	1.01	0.61	-0.19	4	3.9
80	4C	PK4	121.	211.	157.	1.	0.51	0.51	0.41	4	4.0
81	Co1	PK4	100.		1.	1.		0.31	0.51	5	
82	Co2	PK4	145.	400.	625.	703.	1.41	2.91	1.71	5	2.6
83	Co3	PK4	1.	121.	1.	1.	1.21	0.21	0.31	5	3.1
84	Co4	PK4	121.	1.	1.	1.	0.31	0.41	0.11	5	2.4
85	Co5	PK4	91.	157.	1.	1.	0.41	-0.19	-0.19	5	
86	1A	PBS	80.	0.	0.	0.	0.00	0.80	0.30	1	
87	1B	PBS	0.	0.	0.	0.	-0.50	0.10	-0.20	1	
88	1C	PBS	81.	0.	0.	0.		0.10	0.20	1	
89	2A	PBS	36.	0.	0.	0.	-0.40	-0.20	-0.20	2	
90	2B	PBS	0.	0.	0.	0.	0.20	0.20	-0.20	2	
91	2C	PBS	48.	0.	0.	0.	-0.10	-0.30	0.10	2	
92	3A	PBS	120.	0.	0.	0.	-0.60	-0.40	-0.20	3	
93	3B	PBS	0.	0.	0.	0.	0.20	0.20	0.10	3	
94	3C	PBS	0.	0.	0.	0.	0.00	0.00	0.20	3	
95	4A	PBS	0.	0.	0.	0.	0.50	0.30	0.40	4	
96	4B	PBS	100.	0.	0.	0.	0.00	0.00	0.20	4	
97	4C	PBS	99.	0.	0.	0.	0.30	-0.20	0.40	4	
98	Co1	PBS	0.		0.	0.		0.30	0.30	5	
99	Co2	PBS	90.	0.	0.	0.	-0.20	-0.10	0.10	5	
100	Co3	PBS	0.	0.	0.	0.	0.30	0.10	-0.10	5	
101	Co4	PBS	110.	0.	0.	0.	0.00	0.00	0.00	5	
102	Co5	PBS	64.	0.	0.	0.	0.30	-0.20	0.00	5	

DATASET : RABIOP. Chapter five-Cell counts dermal biopsies.

ANTIGEN	NEUTROP 30MIN	EOSINO 30MIN	BASOPH. 30MIN	MONON 30MIN	MAST CELL 30MIN	NEUTROP. 4H	EOSINOPHIL 4H	BASOPHILS. 4H	MONUCLEAR 4H	MAST CELL. 4H	NEUTROP 24H	EOSINOP. 24H	BASOPH. 24H	MONUCLEA 24H	MAST C. 24H
PK1	25	1	0	12	1	23	0	1	25	1	22	2	24	10	5
PK1	51	1	0	64	1	82	0	0	80	4	48	1	28	135	0
PK1	71	1	0	2	1	27	3	2	5	0	27	13	17	6	13
PK2	31	2	5	6	1	158	9	0	3	0	85	31	9	33	56
PK2	13	1	0	82	1	218	3	0	76	0	142	17	28	212	0
PK2	24	5	8	3	0	143	10	1	5	0	59	17	9	24	30
GSG	32	6	1	6	1	52	2	1	3	7	9	19	22	17	28
GSG	17	0	0	58	0	38	0	0	47	0	6	13	20	153	4
GSG	24	7	0	9	3	47	5	0	5	3	6	13	7	16	20
PBS	0	0	0	0	0	0	1	1	1	1	4	4	1	2	
PBS	0	0	0	55	0	0	0	46	0	3	0	3	61	1	
PBS	0	0	0	0	0	0	1	0	2	0	4	7	1	3	

DATASET : BOSTPO. Chapter six-Experiment one.

CASE No.	ANIMAL	ANTIGEN	OEDEMA 15 MIN	OEDEMA 30 MIN	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	ERYTHEMA 48 HOURS	INC.THICK 4 HOURS	INC.THICK 24 HOURS	INC.THICK 48 HOURS	GROUP	MEAN TICK SUSCEPT.
1	8547	PK1	169.	169.	421.	1.	1.	1.61	-0.49	0.01	1	12.87
2	8573	PK1	381.	305.	729.	1.	1.	1.21	-0.29	-0.29	1	19.30
3	8575	PK1	1.	1.	1.	1.	1.	1.31	0.41	0.81	1	9.02
4	8535	PK1	362.	463.	1.	1.	1.	1.11	-0.29	-0.29	1	28.85
5	8571	PK1	257.	271.	625.	1.	1.	2.11	0.11	0.51	1	8.09
6	8539	PK1	1.	241.	1.	1.	1.	1.11	1.51	1.21	1	8.39
7	8519	PK1	169.	273.	1.	1.	1.	0.91	0.11	-0.19	1	8.38
8	8515	PK1	361.	485.	553.	1.	1.	2.11	-1.19	-0.59	1	9.29
9	8567	PK1	441.	481.	1.	1.	1.	1.01	-0.19	0.01	1	18.89
10	8549	PK1	484.	676.	1.	1.	1.	1.31	0.71	0.91	1	19.09
11	8563	PK1	481.	507.	375.	1.	1.	1.61	-0.09	0.01	1	9.51
12	8555	PK1	1.	1.	1.	1.	1.	0.81	0.01	0.01	1	15.99
13	8529	PK1	181.	183.	1.	1.	1.	1.01	0.21	-0.29	1	12.93
14	8597	PK1	401.	1.	1.	1.	1.	1.31	0.41	0.01	1	11.60
15	8569	PK1	305.	400.	286.	1.	1.	0.81	-0.49	-0.69	1	22.81
16	8577	PK1	463.	401.	553.	1.	1.	1.31	0.51	0.01	1	7.79
17	8531	PK1	401.	253.	1.	1.	1.	1.71	0.81	-0.19	1	7.39
18	8525	PK1	421.	343.	1.	1.	1.	0.51	0.21	-0.19	1	25.26
19	8557	PK1	361.	461.	376.	1.	1.	2.51	0.71	0.01	1	8.82
20	8585	PK1	401.	241.	1.	1.	1.	1.11	0.01	-0.19	1	8.87
21	8545	PK1	1.	211.	1.	1.	1.	1.51	0.71	0.71	1	19.00
22	79174	PK1	841.	1477.	4013.	1.		5.81	0.51		2	0.03
23	77366	PK1	601.	1024.	1673.	1.		4.61	2.71		2	3.19
24	76028	PK1	573.	469.	273.	1.		3.01	1.51		2	4.32
25	85507	PK1	931.	929.	897.	1.		2.71	1.71		2	4.80
26	85504	PK1	651.	745.	561.	1.		3.91	0.51		2	0.81
27	8547	PK2	211.	241.	1148.	1.	1.	4.81	0.51	1.01	1	12.87
28	8573	PK2	526.	551.	1021.	1.	1.	2.81	0.51	0.71	1	19.30
29	8575	PK2	273.	307.	553.	290.	1.	3.21	0.81	1.01	1	9.02
30	8535	PK2	961.	993.	1156.	1.	1.	3.61	0.31	-0.19	1	28.85
31	8571	PK2	241.	290.	806.	1.	1.	3.11	0.41	0.41	1	8.09

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 15 MIN	OEDEMA 30 MIN	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	ERYTHEMA 48 HOURS	INC.THICK 4 HOURS	INC.THICK 24 HOURS	INC.THICK 48 HOURS	GROUP	MEAN TICK SUSCEPT.
32	8539	PK2	1.	1.	1.	1.	1.	1.01	0.01	0.21	1	8.39
33	8519	PK2	183.	271.	1.	1.	1.	-0.29	-0.99	-0.29	1	8.38
34	8515	PK2	415.	505.	871.	1.	1.	4.61	0.21	-0.19	1	9.29
35	8567	PK2	341.	601.	1.	1.	1.	2.41	1.21	0.81	1	18.89
36	8549	PK2	421.	484.	1.	1.	1.	3.11	0.81	0.71	1	19.09
37	8563	PK2	529.	530.	737.	1.	1.	3.81	0.61	0.21	1	9.51
38	8555	PK2	1.	1.	1.	1.	1.	1.01	-0.29	0.11	1	15.99
39	8529	PK2	205.	253.	289.	181.	1.	1.91	2.21	0.71	1	12.93
40	8597	PK2	375.	651.	1241.	1.	1.	-0.79	1.81	0.21	1	11.60
41	8569	PK2	375.	622.	419.	1.	1.	2.71	0.71	-0.29	1	22.81
42	8577	PK2	1.	1.	1.	1.	1.	1.31	0.01	-0.49	1	7.79
43	8531	PK2	226.	256.	1.	1.	1.	0.41	-0.29	-0.89	1	7.39
44	8525	PK2	701.	599.	1.	1.	1.	0.61	0.31	-0.29	1	25.26
45	8557	PK2	321.	547.	469.	1.	1.	3.01	0.21	-0.09	1	8.82
46	8585	PK2	341.	400.	1111.	1.	1.	2.71	0.01	-0.69	1	8.87
47	8545	PK2	209.	225.	209.	1.	1.	1.61	0.71	0.01	1	19.00
48	79174	PK2	526.	557.	1.	1.		3.61	2.51		2	0.03
49	77366	PK2	701.	946.	1288.	1.		3.31	0.31		2	3.19
50	76028	PK2	811.	1189.	421.	1.		3.71	2.11		2	4.32
51	85507	PK2	676.	729.	547.	1.		2.81	1.21		2	4.80
52	85504	PK2	461.	343.	1.	1.		1.11	0.81		2	0.81
53	8547	PK3	241.	217.	668.	1.	1.	2.51	-0.09	-0.39	1	12.87
54	8573	PK3	362.	361.	441.	1.	1.	1.71	0.21	0.11	1	19.30
55	8575	PK3	209.	301.	622.	1.	1.	3.31	0.81	0.31	1	9.02
56	8535	PK3	421.	505.	807.	1.	1.	2.71	-0.19	0.31	1	28.85
57	8571	PK3	157.	169.	441.	1.	1.	3.51	0.71	0.31	1	8.09
58	8539	PK3	1.	1.	1.	1.	1.	0.41	0.01	0.11	1	8.39
59	8519	PK3	751.	833.	1021.	958.	1093.	0.71	0.41	0.71	1	8.38
60	8515	PK3	392.	375.	485.	1.	1.	1.41	-0.39	-0.49	1	9.29
61	8567	PK3	442.	625.	1.	1.	1.	1.81	0.81	0.81	1	18.89
62	8549	PK3	1.	121.	1.	1.	1.	1.81	0.71	0.71	1	19.09
63	8563	PK3	341.	331.	358.	1.	1.	2.71	0.01	-0.09	1	9.51
64	8555	PK3	1.	1.	1.	1.	1.	1.11	0.11	0.11	1	15.99
65	8529	PK3	211.	181.	1.	225.	1.	0.91	0.91	0.31	1	12.93
66	8597	PK3	1.	1.	1.	1.	1.	1.31	0.01	-0.19	1	11.60
67	8569	PK3	183.	256.	361.	1.	1.	0.51	-0.39	-0.49	1	22.81
68	8577	PK3	1.	1.	925.	1.	1.	0.01	-0.49	-0.89	1	7.79
69	8531	PK3	155.	307.	1.	1.	1.	0.61	0.21	-1.09	1	7.39
70	8525	PK3	1.	1.	1.	1.	1.	1.41	1.11	0.01	1	25.26
71	8557	PK3	196.	211.	286.	1.	1.	1.41	-0.19	-0.49	1	8.82
72	8585	PK3	392.	441.	1.	1.	1.	1.11	0.01	0.01	1	8.87
73	8545	PK3	1.	256.	341.	1.	1.	1.01	0.21	-0.09	1	19.00
74	79174	PK3	1928.	1693.	1961.	1.		6.91	1.51		2	0.03
75	77366	PK3	842.	1333.	2069.	1.		6.21	1.71		2	3.19
76	76028	PK3	400.	321.	379.	1.		3.61	1.31		2	4.32
77	85507	PK3	530.	576.	601.	1.		2.01	1.11		2	4.80
78	85504	PK3	781.	900.	1522.	1.		2.31	1.91		2	0.81
79	8547	PK4	241.	241.	551.	1.	1.	3.31	0.31	0.61	1	12.87
80	8573	PK4	157.	157.	1.	1.	1.	1.21	-0.29	-0.29	1	19.30
81	8575	PK4	1.	1.	305.	1.	1.	1.51	0.41	0.81	1	9.02
82	8535	PK4	271.	273.	1.	1.	1.	1.11	-0.59	-0.29	1	28.85

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 15 MIN	OEDEMA 30 MIN	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	ERYTHEMA 48 HOURS	INC.THICK 4 HOURS	INC.THICK 24 HOURS	INC.THICK 48 HOURS	GROUP	MEAN TICK SUSCEPT.
83	8571	PK4	197.	197.	421.	1.	1.	2.61	0.01	0.51	1	8.09
84	8539	PK4	1.	1.	1.	1.	1.	0.51	0.41	0.21	1	8.39
85	8519	PK4	1.	1.	1.	1.	1.	0.01	0.21	0.01	1	8.38
86	8515	PK4	141.	253.	751.	1.	1.	3.51	-0.09	-0.29	1	9.29
87	8567	PK4	628.	829.	1.	1.	1.	1.81	0.71	0.61	1	18.89
88	8549	PK4	324.	343.	838.	1.	1.	2.51	0.51	0.61	1	19.09
89	8563	PK4	289.	256.	651.	1.	1.	2.31	0.01	-0.19	1	9.51
90	8555	PK4	183.	1.	1.	1.	1.	1.21	-0.29	-0.39	1	15.99
91	8529	PK4	144.	122.	495.	1.	1.	1.51	0.41	0.41	1	12.93
92	8597	PK4	151.	1.	1.	1.	1.	1.21	0.11	-0.29	1	11.60
93	8569	PK4	256.	343.	239.	1.	1.	1.41	0.21	-0.09	1	22.81
94	8577	PK4	1.	1.	1.	1.	1.	0.61	-0.49	-0.79	1	7.79
95	8531	PK4	157.	1.	1.	1.	1.	1.61	0.31	-0.19	1	7.39
96	8525	PK4	1.	1.	1.	1.	1.	0.91	0.61	0.01	1	25.26
97	8557	PK4	197.	196.	281.	1.	1.	1.11	-0.49	-0.69	1	8.82
98	8585	PK4	324.	324.	421.	1.	1.	0.71	0.21	0.01	1	8.87
99	8545	PK4	133.	289.	397.	1.	1.	1.51	0.81	0.11	1	19.00
100	79174	PK4	991.	1121.	989.	1.		2.81	0.71		2	0.03
101	77366	PK4	649.	1111.	1121.	1.		2.61	0.71		2	3.19
102	76028	PK4	595.	183.	211.	1.		1.51	0.41		2	4.32
103	85507	PK4	463.	553.	505.	1.		2.51	1.91		2	4.80
104	85504	PK4	651.	1025.	1177.	1.		2.11	0.91		2	0.81
105	8547	GSG-10	183.	211.	375.	1.	1.	0.71	-0.89	-0.19	1	12.87
106	8573	GSG-10	401.	505.	1.	1.	1.	1.01	0.01	-0.59	1	19.30
107	8575	GSG-10	256.	289.	289.	1.	1.	1.11	0.31	1.31	1	9.02
108	8535	GSG-10	289.	375.	1.	1.	1.	0.71	-0.29	-0.39	1	28.85
109	8571	GSG-10	1.	235.	307.	1.	1.	1.41	-0.09	0.41	1	8.09
110	8539	GSG-10	155.	1.	1.	1.	1.	0.31	0.41	0.01	1	8.39
111	8519	GSG-10	267.	271.	1.	1.	1.	1.01	0.41	0.31	1	8.38
112	8515	GSG-10	324.	271.	307.	1.	1.	1.41	-0.49	-0.59	1	9.29
113	8567	GSG-10	343.	1.	1.	1.	1.	0.71	0.31	0.11	1	18.89
114	8549	GSG-10	400.	400.	1.	1.	1.	1.01	0.21	0.31	1	19.09
115	8563	GSG-10	507.	625.	851.	1.	1.	3.41	0.71	0.71	1	9.51
116	8555	GSG-10	211.	1.	1.	1.	1.	0.91	-0.29	-0.29	1	15.99
117	8529	GSG-10	170.	169.	419.	1.	1.	0.41	-0.19	-0.29	1	12.93
118	8597	GSG-10	1.	1.	1.	1.	1.	2.11	0.01	-0.19	1	11.60
119	8569	GSG-10	289.	256.	341.	1.	1.	0.81	-0.19	-0.29	1	22.81
120	8577	GSG-10	343.	1.	209.	1.	1.	0.71	0.21	0.01	1	7.79
121	8531	GSG-10	324.	305.	1.	1.	1.	2.01	0.01	0.01	1	7.39
122	8525	GSG-10	1.	1.	1.	1.	1.	0.31	0.51	0.31	1	25.26
123	8557	GSG-10	196.	343.	341.	1.	1.	1.41	-0.39	-0.59	1	8.82
124	8585	GSG-10	397.	421.	481.	1.	1.	1.51	-0.19	-0.29	1	8.87
125	8545	GSG-10	205.	211.	530.	1.	1.	0.71	0.11	-0.09	1	19.00
126	79174	GSG-10	838.	1024.	721.	1.		4.91	1.41		2	0.03
127	77366	GSG-10	673.	1429.	2022.	1.		3.01	0.51		2	3.19
128	76028	GSG-10	421.	379.	1.	1.		1.51	0.51		2	4.32
129	85507	GSG-10	649.	784.	1.	1.		2.51	1.71		2	4.80
130	85504	GSG-10	622.	676.	727.	1.		1.81	0.51		2	0.81
131	8547	100-GS	145.	133.	1.	1.	1.	0.01	-0.49	-0.09	1	12.87
132	8573	100-GS	183.	183.	1.	1.	1.	-0.59	-0.79	-0.79	1	19.30
133	8575	100-GS	144.	145.	1.	1.	1.	0.71	-0.09	-0.09	1	9.02

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 15 MIN	OEDEMA 30 MIN	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	ERYTHEMA 48 HOURS	INC.THICK 4 HOURS	INC.THICK 24 HOURS	INC.THICK 48 HOURS	GROUP	MEAN TICK SUSCEPT.
134	8535	100-GS	1.	1.	1.	1.	1.	0.61	-0.39	0.01	1	28.85
135	8571	100-GS	183.	1.	1.	1.	1.	0.61	0.01	-0.09	1	8.09
136	8539	100-GS	1.	1.	1.	1.	1.	0.31	-0.09	0.31	1	8.39
137	8519	100-GS	1.	1.	1.	1.	1.	0.21	0.11	-0.09	1	8.38
138	8515	100-GS	157.	145.	1.	1.	1.	0.61	-0.69	-0.39	1	9.29
139	8567	100-GS	241.	381.	1.	1.	1.	2.01	0.61	0.61	1	18.89
140	8549	100-GS	122.	111.	1.	1.	1.	0.91	0.41	0.31	1	19.09
141	8563	100-GS	358.	421.	501.	1.	1.	1.21	-0.29	-0.99	1	9.51
142	8555	100-GS	133.	1.	1.	1.	1.	0.51	0.01	0.11	1	15.99
143	8529	100-GS	1.	1.	1.	1.	1.	0.61	0.11	0.11	1	12.93
144	8597	100-GS	1.	1.	1.	1.	1.	3.01	0.61	0.11	1	11.60
145	8569	100-GS	181.	155.	1.	1.	1.	-0.19	0.01	-0.09	1	22.81
146	8577	100-GS	1.	1.	1.	1.	1.	0.81	-0.19	-0.49	1	7.79
147	8531	100-GS	144.	196.	1.	1.	1.	0.41	-0.09	-0.19	1	7.39
148	8525	100-GS	181.	290.	1.	1.	1.	2.11	0.51	0.31	1	25.26
149	8557	100-GS	121.	145.	157.	1.	1.	0.21	-0.19	-0.49	1	8.82
150	8585	100-GS	183.	271.	141.	1.	1.	0.71	-0.19	0.01	1	8.87
151	8545	100-GS	121.	197.	1.	1.	1.	0.71	0.61	0.11	1	19.00
152	79174	100-GS	981.	929.	1321.	1.		3.01	0.61		2	0.03
153	77366	100-GS	144.	111.	1.	1.		1.61	0.71		2	3.19
154	76028	100-GS	421.	484.	343.	1.		1.11	1.11		2	4.32
155	85507	100-GS	225.	1.	1.	1.		-0.09	0.21		2	4.80
156	85504	100-GS	343.	1.	2445.	1.		3.01	0.01		2	0.81
157	8547	LAR20	321.	379.	547.	1.	1.	1.51	0.51	1.31	1	12.87
158	8573	LAR20	751.	811.	833.	1.	1.	1.81	-0.09	-0.69	1	19.30
159	8575	LAR20	324.	421.	900.	183.	1.	4.81	1.21	1.91	1	9.02
160	8535	LAR20	438.	514.	649.	1.	1.	2.91	-0.69	-0.89	1	28.85
161	8571	LAR20	1.	1.	973.	109.	157.	4.21	0.61	2.11	1	8.09
162	8539	LAR20	253.	273.	196.	144.	1.	1.21	1.01	0.51	1	8.39
163	8519	LAR20	144.	305.	1.	111.	1.	1.11	0.71	0.81	1	8.38
164	8515	LAR20	183.	442.	1121.	169.	1.	5.11	0.81	-0.39	1	9.29
165	8567	LAR20	729.	958.	1.	419.	1.	1.91	1.81	1.91	1	18.89
166	8549	LAR20	551.	703.	1086.	145.	210.	3.11	1.51	1.21	1	19.09
167	8563	LAR20	463.	919.	1849.	225.	1.	6.41	2.71	0.81	1	9.51
168	8555	LAR20	1.	1.	1.	1.	1.	0.91	-0.19	0.11	1	15.99
169	8529	LAR20	239.	362.	1.	316.	1.	2.11	2.51	1.11	1	12.93
170	8597	LAR20	1.	1.	1.	1.	1.	-0.09	-0.09	-0.59	1	11.60
171	8569	LAR20	381.	631.	931.	1.	1.	3.31	0.41	0.11	1	22.81
172	8577	LAR20	1.	1.	1.	1.	1.	1.01	0.81	0.11	1	7.79
173	8531	LAR20	211.	273.	169.	1.	1.	1.91	-0.09	-0.79	1	7.39
174	8525	LAR20	526.	484.	1.	1.	1.	0.71	0.31	0.01	1	25.26
175	8557	LAR20	401.	484.	551.	1.	1.	6.31	0.91	0.91	1	8.82
176	8585	LAR20	461.	526.	929.	1.	1.	2.51	0.71	0.31	1	8.87
177	8545	LAR20	307.	484.	645.	1.	1.	2.71	1.01	0.11	1	19.00
178	79174	LAR20	1441.	2146.	1.	89.		2.61	0.31		2	0.03
179	77366	LAR20	457.	755.	1296.	1.		4.71	2.01		2	3.19
180	76028	LAR20	589.	691.	601.	1.		3.51	1.81		2	4.32
181	85507	LAR20	901.	1156.	1.	1.		2.01	1.61		2	4.80
182	85504	LAR20	1086.	1407.	573.	1.		2.41	1.91		2	0.81
183	8547	L-200	157.	211.	421.	1.	1.	1.61	0.11	1.01	1	12.87
184	8573	L-200	239.	289.	1.	1.	1.	0.51	-0.39	-0.09	1	19.30

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 15 MIN	OEDEMA 30 MIN	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	ERYTHEMA 48 HOURS	INC.THICK 4 HOURS	INC.THICK 24 HOURS	INC.THICK 48 HOURS	GROUP	MEAN TICK SUSCEPT.
185	8575	L-200	289.	381.	341.	1.	1.	2.71	0.51	1.01	1	9.02
186	8535	L-200	307.	421.	1.	1.	1.	1.81	-0.79	-0.09	1	28.85
187	8571	L-200	235.	197.	256.	1.	1.	1.71	0.21	0.91	1	8.09
188	8539	L-200	267.	381.	1.	1.	1.	1.31	0.41	0.61	1	8.39
189	8519	L-200	197.	157.	1.	1.	1.	0.71	0.61	0.51	1	8.38
190	8515	L-200	1.	290.	400.	1.	1.	1.61	-0.79	-0.89	1	9.29
191	8567	L-200	485.	625.	1.	1.	1.	1.51	0.11	0.21	1	18.89
192	8549	L-200	361.	442.	1.	1.	1.	1.91	0.41	0.51	1	19.09
193	8563	L-200	257.	181.	169.	1.	1.	1.31	0.21	-0.19	1	9.51
194	8555	L-200	1.	1.	1.	1.	1.	0.61	-0.49	-0.39	1	15.99
195	8529	L-200	183.	290.	1.	1.	1.	0.31	0.11	0.01	1	12.93
196	8597	L-200	343.	419.	1.	1.	1.	0.51	0.01	-0.39	1	11.60
197	8569	L-200	324.	441.	529.	1.	1.	0.61	-0.49	0.01	1	22.81
198	8577	L-200	1.	1.	1.	1.	1.	1.01	0.11	-0.39	1	7.79
199	8531	L-200	121.	256.	343.	1.	1.	1.71	-0.19	-0.19	1	7.39
200	8525	L-200	703.	1.	1.	1.	1.	1.11	-0.19	-0.99	1	25.26
201	8557	L-200	183.	155.	209.	1.	1.	0.51	-0.49	-0.99	1	8.82
202	8585	L-200	307.	343.	573.	1.	1.	1.71	0.11	-0.09	1	8.87
203	8545	L-200	145.	170.	1.	1.	1.	0.41	0.21	0.01	1	19.00
204	79174	L-200	776.	2689.	1.	1.		3.21	0.31		2	0.03
205	77366	L-200	813.	1436.	1.	1.		3.61	1.31		2	3.19
206	76028	L-200	196.	755.	625.	1.		2.01	0.01		2	4.32
207	85507	L-200	419.	505.	505.	1.		1.51	1.01		2	4.80
208	85504	L-200	379.	485.	1361.	1.		2.41	1.01		2	0.81
209	8547	PBS	169.	1.	1.	1.	1.	-0.09	-0.19	0.21	1	12.87
210	8573	PBS	1.	1.	1.	1.	1.	0.51	-0.29	-0.39	1	19.30
211	8575	PBS	1.	1.	1.	1.	1.	0.21	-0.19	0.11	1	9.02
212	8535	PBS	1.	1.	1.	1.	1.	0.81	-0.09	0.11	1	28.85
213	8571	PBS	1.	1.	1.	1.	1.	-0.29	-0.89	0.11	1	8.09
214	8539	PBS	1.	256.	1.	1.	1.	0.31	-0.09	0.31	1	8.39
215	8519	PBS	157.	1.	1.	1.	1.	0.21	0.01	0.01	1	8.38
216	8515	PBS	1.	1.	1.	1.	1.	-0.49	-1.39	-1.39	1	9.29
217	8567	PBS	1.	1.	1.	1.	1.	0.61	0.01	0.21	1	18.89
218	8549	PBS	157.	91.	1.	1.	1.	1.01	0.41	0.71	1	19.09
219	8563	PBS	1.	1.	1.	1.	1.	0.01	0.01	-0.69	1	9.51
220	8555	PBS	1.	1.	1.	1.	1.	1.11	0.11	0.41	1	15.99
221	8529	PBS	1.	1.	1.	1.	1.	0.01	-0.29	-0.19	1	12.93
222	8597	PBS	1.	1.	1.	1.	1.	1.91	-0.39	-0.79	1	11.60
223	8569	PBS	1.	1.	1.	1.	1.	0.01	0.11	-0.19	1	22.81
224	8577	PBS	1.	1.	1.	1.	1.	-0.29	-0.49	-0.69	1	7.79
225	8531	PBS	1.	1.	1.	1.	1.	0.71	-0.39	-0.39	1	7.39
226	8525	PBS	1.	1.	1.	1.	1.	0.91	1.11	0.61	1	25.26
227	8557	PBS	73.	1.	1.	1.	1.	0.01	-0.59	-0.59	1	8.82
228	8585	PBS	1.	1.	1.	1.	1.	0.51	-0.29	-0.39	1	8.87
229	8545	PBS	1.	1.	1.	1.	1.	0.01	-0.09	-0.49	1	19.00
230	79174	PBS	1.	1.	1.	1.		1.41	0.71		2	0.03
231	77366	PBS	144.	1.	1.	1.		0.81	-0.09		2	3.19
232	76028	PBS	1.	1.	1.	1.		0.11	0.21		2	4.32
233	85507	PBS	89.	1.	157.	1.		0.81	0.61		2	4.80
234	85504	PBS	1.	1.	726.	1.		0.81	0.31		2	0.81

DATASET : BEHAVI. Chapter six. Experiment two

CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 Min DIL 0	TOT.REACT 4H DIL 0	% SUSCEPT. TO TICKS	OEDEMA 30 Min DIL1	TOT.REACT 4H DIL1	OEDEMA 30 Min DIL2	TOT.REACT 4H DIL2	OEDEMA 30 Min DIL3	TOT.REACT 4H DIL3	TRANSFORD. SUSCEPBTY	DOSE 500 AREA 30min	DOSE 10mm AREA 30mi
n														
1	8545	PK1	925.	975.24	3.30	221.	381.78	180.	0.91	1.	0.11	1.35	0.90	2.64
2	8545	PK2	840.	3222.18	3.30	484.	320.76	1.	0.51	1.	79.30	1.35		
3	8545	PK3	506.	1.41	3.30	240.	0.51	1.	0.31	1.	-0.19	1.35		
4	8545	GSG	837.	687.60	3.30	528.	0.91	1.	0.31	1.	0.11	1.35	0.98	2.59
5	8563	PK1	1.	-78.30	4.06	1.	0.71	1.	0.51	1.	0.01	1.42		
6	8563	PK2	782.	0.51	4.06	1.	0.51	1.	0.21	1.	0.21	1.42		
7	8563	PK3	142.	0.51	4.06	1.	0.21	1.	0.21	1.	0.21	1.42		
8	8563	GSG	750.	0.81	4.06	340.	0.31	156.	0.01	100.	0.11	1.42	0.73	3.03
9	8535	PK1	783.		6.26	644.		210.		120.		1.58	1.25	3.27
10	8535	PK2	462.		6.26	924.		225.				1.58		
11	8535	PK3	504.		6.26							1.58		
12	8535	GSG	1008.		6.26	812.						1.58	2.59	5.09
13	8597	PK1	980.		4.28	316.		306.		110.		1.44	1.23	3.09
14	8597	PK2	900.		4.28	650.		420.				1.44		
15	8597	PK3	418.		4.28							1.44		
16	8597	GSG	552.		4.28	360.		272.		154.		1.44	0.21	4.03
17	8567	PK1			8.94	405.						1.73		
18	8567	PK2	1344.		8.94							1.73		
19	8567	PK3	378.		8.94							1.73		
20	8567	GSG	289.		8.94	252.		182.		81.		1.73	-2.81	4.25
21	8549	PK1	550.	1647.03	3.88	378.	0.11	1.	0.71	1.	0.11	1.40	0.18	2.60
22	8549	PK2	460.	1270.62	3.88	432.	-0.19	1.	0.31	1.	0.21	1.40		
23	8549	PK3	380.	635.25	3.88	168.	0.31	1.	0.01	1.	0.31	1.40		
24	8549	GSG	378.	488.40	3.88	399.	584.43	208.	0.61	1.	0.21	1.40	-0.42	3.29
25	8575	PK1	1440.	2425.20	3.52	725.	500.61	572.	0.11	196.	182.25	1.37	2.10	3.36
26	8575	PK2	1056.	0.71	3.52	484.	0.91	221.	0.61	1.	0.41	1.37		
27	8575	PK3	420.	0.81	3.52	120.	0.41	1.	0.11	1.	0.41	1.37		
28	8575	GSG	1122.	1.61	3.52	783.	1.21	256.	0.21	154.	0.21	1.37	1.73	3.16
29	8557	PK1	810.	1.81	7.94	756.	0.51	1.	0.41	1.	0.41	1.68	1.16	2.70
30	8557	PK2	1088.	1.01	7.94	1008.	0.41	224.	0.41	1.	0.31	1.68		
31	8557	PK3	504.	1.31	7.94	196.	0.21	1.	0.21	1.	0.31	1.68		
32	8557	GSG	756.	1.61	7.94	600.	1.01	288.	0.81	132.	-0.09	1.68	1.24	3.49
33	8573	PK1	1020.	0.81	2.82	1.	1.31	1.	1.01	1.	0.71	1.30	0.70	2.30
34	8573	PK2	1218.	1.91	2.82	988.	0.81	306.	0.41	1.	0.21	1.30		
35	8573	PK3	638.	1.21	2.82	1.	0.21	1.	0.31	1.	0.21	1.30		
36	8573	GSG	625.	1.91	2.82	460.	0.91	195.	0.31	132.	0.31	1.30	0.66	3.47
37	8569	PK1	832.	1.11	4.24	525.	1.01	493.	0.41	195.	0.41	1.43	1.56	4.08
38	8569	PK2	850.	907.20	4.24	624.	1172.08	225.	0.91	195.	0.41	1.43		
39	8569	PK3	418.	1025.73	4.24	154.	0.81	1.	0.91	1.	0.31	1.43		
40	8569	GSG	756.	0.51	4.24	500.	0.51	240.	0.21	154.	0.51	1.43	1.08	3.45
41	8519	PK1	506.	942.24	2.70	360.	0.81	1.	0.21	1.	0.11	1.28	-0.01	2.60
42	8519	PK2	576.	1356.75	2.70	361.	0.41	156.	0.31	156.	0.01	1.28		
43	8519	PK3	460.	377.40	2.70	378.	0.01	1.	-0.19	143.	-0.19	1.28		
44	8519	GSG	648.	0.81	2.70	399.	0.01	132.	0.21	90.	0.01	1.28	0.56	3.08
45	8525	PK1	1122.	1.41	3.22	528.	1.31	210.	0.71	1.	0.41	1.34	1.41	2.74
46	8525	PK2	1224.	1.21	3.22	550.	0.71	1.	0.21	182.	-0.39	1.34		
47	8525	PK3	841.	0.51	3.22	1.	-0.39	1.	-0.09	1.	0.81	1.34		
48	8525	GSG	840.	1.71	3.22	1.	0.71	182.	0.21	132.	0.11	1.34	0.41	2.94

DATASET : ADQRES. Chapter seven.

CASE No.	ANIMAL	TEST	EXPOSURES TO TICKS	STATUS	ANTIGEN	OEDEMA 30 min	ERYTHEMA 4h	ERYTHEMA 24h	4H INC. THICKNESS	24H INC. THICKNESS	% SUSCEPT TO TICKS	NORMALSKIN THICKNESS	END POINT TITRE A	END POINT TITRE B
1	8664	1	0	1	P1	1.	1.	1.	0.21	-0.49	12.10	4.70	3.1	5.1
2	8664	1	0	1	P2	1.	1.	1.	0.51	0.21	12.10	5.00	3.1	5.1
3	8664	1	0	1	P3	11.	13.	1.	1.21	0.31	12.10	4.30	3.1	5.1
4	8664	1	0	1	GSG	12.	1.	1.	0.91	-0.79	12.10	4.60	3.1	5.1
5	8664	1	0	1	PBS	1.	1.	1.	0.51	0.11	12.10	6.00	3.1	5.1
6	8695	1	0	1	P1	8.	12.	10.	0.81	0.51	17.30	5.00	3.2	5.4
7	8695	1	0	1	P2	1.	1.	13.	0.21	0.51	17.30	5.30	3.2	5.4
8	8695	1	0	1	P3	7.	5.	1.	1.21	1.41	17.30	4.80	3.2	5.4
9	8695	1	0	1	GSG	1.	14.	1.	2.11	1.21	17.30	4.40	3.2	5.4
10	8695	1	0	1	PBS	1.	1.	1.	0.61	0.11	17.30	4.90	3.2	5.4
11	8670	1	0	1	P1	10.	10.	10.	0.81	0.51	6.60	4.20	2.8	4.0
12	8670	1	0	1	P2	9.	13.	11.	-0.29	0.01	6.60	5.60	2.8	4.0
13	8670	1	0	1	P3	1.	10.	1.	0.71	0.41	6.60	4.50	2.8	4.0
14	8670	1	0	1	GSG	10.	15.	1.	1.51	0.51	6.60	4.80	2.8	4.0
15	8670	1	0	1	PBS	10.	1.	1.	0.01	-0.29	6.60	5.50	2.8	4.0
16	8699	1	0	1	P1	1.	10.	11.	0.21	0.71	4.26	4.50	3.6	5.4
17	8699	1	0	1	P2	1.	11.	1.	0.41	0.71	4.26	4.80	3.6	5.4
18	8699	1	0	1	P3	12.	12.	1.	0.61	0.31	4.26	4.30	3.6	5.4
19	8699	1	0	1	GSG	20.	15.	1.	1.01	0.11	4.26	4.30	3.6	5.4
20	8699	1	0	1	PBS	1.	1.	1.	0.21	0.31	4.26	5.20	3.6	5.4
21	6101	1	0	1	P1	10.	12.	12.	0.21	0.41	10.80	5.50	4.3	5.6
22	6101	1	0	1	P2	1.	12.	9.	0.51	0.41	10.80	6.00	4.3	5.6
23	6101	1	0	1	P3	12.	12.	1.	1.11	0.21	10.80	5.30	4.3	5.6
24	6101	1	0	1	GSG	1.	1.	1.	0.01	0.11	10.80	5.40	4.3	5.6
25	6101	1	0	1	PBS	1.	1.	1.	-0.19	-0.59	10.80	6.60	4.3	5.6
26	869N	1	0	1	P1	9.	10.	8.	0.01	0.41	6.92	4.60	2.2	4.8
27	869N	1	0	1	P2	12.	14.	1.	1.21	1.01	6.92	5.00	2.2	4.8
28	869N	1	0	1	P3	10.	9.	1.	0.61	0.11	6.92	4.40	2.2	4.8
29	869N	1	0	1	GSG	18.	16.	14.	1.91	1.31	6.92	5.00	2.2	4.8
30	869N	1	0	1	PBS	1.	1.	1.	0.21	0.11	6.92	6.10	2.2	4.8
31	8664	2	1	2	P1	25.	18.	17.	2.71	2.31	2.44	5.10	5.1	5.8
32	8664	2	1	2	P2	34.	18.	18.	3.21	2.31	2.44	5.50	5.1	5.8
33	8664	2	1	2	P3	23.	14.	19.	2.61	2.41	2.44	4.50	5.1	5.8
34	8664	2	1	2	GSG	39.	28.	19.	5.21	4.61	2.44	4.70	5.1	5.8
35	8664	2	1	2	PBS	1.	1.	1.	-0.49	-0.99	2.44	6.80	5.1	5.8
36	8695	2	1	2	P1	23.	18.	22.	1.31	4.41	4.18	4.50	5.4	5.5
37	8695	2	1	2	P2	17.	15.	26.	2.31	5.11	4.18	5.40	5.4	5.5
38	8695	2	1	2	P3	22.	16.	17.	2.21	3.11	4.18	5.20	5.4	5.5
39	8695	2	1	2	GSG	30.	28.	31.	3.91	7.51	4.18	5.50	5.4	5.5
40	8695	2	1	2	PBS	1.	1.	1.	0.41	0.11	4.18	5.20	5.4	5.5
41	8670	2	1	2	P1	29.	14.	15.	1.01	2.71	0.52	4.30	4.0	6.2
42	8670	2	1	2	P2	25.	18.	19.	2.01	2.51	0.52	5.00	4.0	6.2
43	8670	2	1	2	P3	23.	18.	21.	1.61	2.11	0.52	5.70	4.0	6.2
44	8670	2	1	2	GSG	29.	22.	24.	2.71	5.21	0.52	4.80	4.0	6.2
45	8670	2	1	2	PBS	1.	1.	1.	0.51	-0.09	0.52	5.50	4.0	6.2
46	8699	2	1	2	P1	14.	13.	13.	1.61	2.11	1.88	4.30	5.4	5.7
47	8699	2	1	2	P2	25.	15.	17.	1.31	2.21	1.88	5.00	5.4	5.7
48	8699	2	1	2	P3	19.	12.	15.	1.11	1.51	1.88	5.20	5.4	5.7
49	8699	2	1	2	GSG	31.	24.	20.	3.21	3.71	1.88	4.60	5.4	5.7
50	8699	2	1	2	PBS	1.	1.	1.	-0.19	-0.09	1.88	4.70	5.4	5.7

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CASE No.	ANIMAL	TEST	EXPOSURES TO TICKS	STATUS	ANTIGEN	OEDEMA 30 min	ERYTHEMA 4h	ERYTHEMA 24h	4H INC. THICKNESS	24H INC. THICKNESS	% SUSCEPT TO TICKS	NORMALSKIN THICKNESS	END POINT TITRE A	END POINT TITRE B
51	6101	2	1	2	P1	24.	18.	14.	2.61	2.11	4.48	5.50	5.6	7.3
52	6101	2	1	2	P2	26.	17.	20.	2.41	2.01	4.48	6.70	5.6	7.3
53	6101	2	1	2	P3	19.	14.	16.	1.31	1.51	4.48	7.20	5.6	7.3
54	6101	2	1	2	GSG	29.	21.	26.	3.61	5.01	4.48	5.80	5.6	7.3
55	6101	2	1	2	PBS	1.	1.	1.	-0.09	0.21	4.48	6.20	5.6	7.3
56	869M	2	1	2	P1	19.	17.	14.	2.11	1.81	0.50	4.60	4.8	5.0
57	869M	2	1	2	P2	25.	17.	13.	1.71	1.01	0.50	6.00	4.8	5.0
58	869M	2	1	2	P3	27.	17.	16.	1.81	1.81	0.50	5.50	4.8	5.0
59	869M	2	1	2	GSG	32.	31.	23.	5.31	4.81	0.50	4.80	4.8	5.0
60	869M	2	1	2	PBS	9.	1.	1.	0.21	0.21	0.50	4.90	4.8	5.0
61	8660	1	0	2	P1	1.	1.	1.	0.91	0.01	24.40	4.60	3.1	4.8
62	8660	1	0	2	P2	11.	13.	1.	0.91	-0.19	24.40	5.40	3.1	4.8
63	8660	1	0	2	P3	1.	1.	1.	0.51	-0.09	24.40	5.80	3.1	4.8
64	8660	1	0	2	GSG	17.	12.	1.	1.11	0.21	24.40	4.40	3.1	4.8
65	8660	1	0	2	PBS	1.	1.	1.	0.21	-0.49	24.40	5.00	3.1	4.8
66	6103	1	0	5	P1	11.	9.	11.	0.41	0.11		4.60	2.5	2.9
67	6103	1	0	5	P2	1.	1.	1.	0.61	0.11		5.00	2.5	2.9
68	6103	1	0	5	P3	1.	1.	1.	0.31	0.01		5.30	2.5	2.9
69	6103	1	0	5	GSG	20.	1.	1.	0.71	0.31		4.50	2.5	2.9
70	6103	1	0	5	PBS	1.	1.	1.	0.11	-0.19		4.60	2.5	2.9
71	8664	3	2	3	P1	37.	42.	16.	7.41	1.51	2.66	5.10	5.8	6.8
72	8664	3	2	3	P2	40.	42.	1.	2.31	1.61	2.66	5.70	5.8	6.8
73	8664	3	2	3	P3	33.	27.	14.	2.71	1.41	2.66	5.30	5.8	6.8
74	8664	3	2	3	GSG	48.	41.	15.	1.81	1.11	2.66	5.60	5.8	6.8
75	8664	3	2	3	PBS	1.	1.	1.			2.66		5.8	6.8
76	8695	3	2	3	P1	32.	30.	20.	3.51	2.01	5.00	6.10	5.5	5.4
77	8695	3	2	3	P2	28.	30.	19.	3.21	2.11	5.00	6.80	5.5	5.4
78	8695	3	2	3	P3	26.	33.	1.	3.21	1.01	5.00	6.00	5.5	5.4
79	8695	3	2	3	GSG	38.	39.	29.	3.91	3.51	5.00	6.50	5.5	5.4
80	8695	3	2	3	PBS	1.	1.	1.			5.00		5.5	5.4
81	8670	3	2	3	P1	31.	35.	14.	3.41	1.41	0.80	5.00	6.2	6.7
82	8670	3	2	3	P2	41.	45.	24.	4.31	2.31	0.80	6.70	6.2	6.7
83	8670	3	2	3	P3	33.	37.	17.	4.41	2.21	0.80	5.60	6.2	6.7
84	8670	3	2	3	GSG	38.	44.	28.	6.01	6.01	0.80	6.00	6.2	6.7
85	8670	3	2	3	PBS	1.	1.	1.			0.80		6.2	6.7
86	8699	3	2	3	P1	30.	25.	15.	3.01	1.51	5.40	5.00	5.7	5.9
87	8699	3	2	3	P2	38.	40.	21.	2.91	1.61	5.40	6.20	5.7	5.9
88	8699	3	2	3	P3	26.	28.	15.	2.91	0.61	5.40	5.30	5.7	5.9
89	8699	3	2	3	GSG	42.	35.	21.	3.81	2.61	5.40	5.40	5.7	5.9
90	8699	3	2	3	PBS	1.	1.	1.			5.40		5.7	5.9
91	6101	3	2	3	P1	31.	27.	15.	3.91	1.21	8.50	6.10	7.3	9.8
92	6101	3	2	3	P2	38.	31.	22.	4.81	1.71	8.50	6.80	7.3	9.8
93	6101	3	2	3	P3	33.	37.	19.	3.01	0.91	8.50	6.90	7.3	9.8
94	6101	3	2	3	GSG	33.	33.	25.	5.41	3.01	8.50	6.60	7.3	9.8
95	6101	3	2	3	PBS	1.	1.	1.			8.50		7.3	9.8
96	869M	3	2	3	P1	43.	30.	16.	3.51	1.71	6.46	5.50	5.0	5.7
97	869M	3	2	3	P2	32.	34.	19.	3.41	1.71	6.46	6.60	5.0	5.7
98	869M	3	2	3	P3	32.	27.	22.	4.01	2.11	6.46	5.50	5.0	5.7
99	869M	3	2	3	GSG	38.	35.	29.	4.31	3.51	6.46	7.00	5.0	5.7
100	869M	3	2	3	PBS	1.	1.	1.			6.46		5.0	5.7
101	8660	2	1	3	P1	27.	27.	17.	3.31	2.01	7.88	4.90	4.8	5.9

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CASE No.	ANIMAL	TEST	EXPOSURES TO TICKS	STATUS	ANTIGEN	OEDEMA 30 min	ERYTHEMA 4h	ERYTHEMA 24h	4H INC. THICKNESS	24H INC. THICKNESS	% SUSCEPT TO TICKS	NORMALSKIN THICKNESS	END POINT TITRE A	END POINT TITRE B
102	8660	2	1	3	P2	30.	31.	16.	3.51	1.41	7.88	5.60	4.8	5.9
103	8660	2	1	3	P3	22.	25.	14.	3.01	1.41	7.88	4.60	4.8	5.9
104	8660	2	1	3	GSG	33.	38.	19.	3.91	1.91	7.88	5.10	4.8	5.9
105	8660	2	1	3	PBS	1.	1.	1.			7.88		4.8	5.9
106	6103	2	0	5	P1	1.	1.	1.	0.21	1.01		5.40	2.9	3.8
107	6103	2	0	5	P2	1.	17.	1.	0.51	0.11		6.50	2.9	3.8
108	6103	2	0	5	P3	1.	14.	12.	0.01	1.01		5.60	2.9	3.8
109	6103	2	0	5	GSG	27.	17.	1.	0.51	0.51		6.10	2.9	3.8
110	6103	2	0	5	PBS	1.	1.	1.					2.9	3.8
111	8662	1	0	3	P1	1.	1.	13.	0.81	0.81	4.28	6.00	2.9	6.2
112	8662	1	0	3	P2	1.	18.	1.	1.91	0.41	4.28	6.60	2.9	6.2
113	8662	1	0	3	P3	1.	18.	12.	1.81	0.31	4.28	6.10	2.9	6.2
114	8662	1	0	3	GSG	25.	21.	16.	2.61	1.61	4.28	6.40	2.9	6.2
115	8662	1	0	3	PBS	1.	1.	1.			4.28		2.9	6.2
116	8664	4	3	4	P1	39.	18.	18.	3.01	2.71	0.76	5.30	6.8	6.5
117	8664	4	3	4	P2	40.	30.	19.	4.41	2.91	0.76	6.70	6.8	6.5
118	8664	4	3	4	P3	30.	28.	17.	2.21	2.11	0.76	6.00	6.8	6.5
119	8664	4	3	4	GSG	60.	31.	22.	3.91	2.41	0.76	8.10	6.8	6.5
120	8664	4	3	4	PBS	1.	1.	1.	-0.39	-0.29	0.76	8.70	6.8	6.5
121	8695	4	3	4	P1	30.	24.	26.	3.01	1.61	1.50	7.10	5.4	5.1
122	8695	4	3	4	P2	34.	27.	21.	3.11	2.31	1.50	6.90	5.4	5.1
123	8695	4	3	4	P3	31.	21.	21.	3.41	1.71	1.50	6.10	5.4	5.1
124	8695	4	3	4	GSG	32.	36.	36.	4.31	4.51	1.50	7.30	5.4	5.1
125	8695	4	3	4	PBS	1.	1.	1.	0.61	0.41	1.50	6.70	5.4	5.1
126	8670	4	3	4	P1	31.	31.	16.	2.01	1.41	0.22	5.80	6.7	7.8
127	8670	4	3	4	P2	44.	27.	16.	2.41	0.81	0.22	6.60	6.7	7.8
128	8670	4	3	4	P3	37.	32.	1.	3.11	1.01	0.22	7.50	6.7	7.8
129	8670	4	3	4	GSG	46.	28.	24.	3.41	2.11	0.22	6.30	6.7	7.8
130	8670	4	3	4	PBS	1.	1.	1.	0.91	0.01	0.22	8.20	6.7	7.8
131	8699	4	3	4	P1	29.	25.	1.	2.01	1.01	0.32	6.00	5.9	5.7
132	8699	4	3	4	P2	37.	29.	18.	4.31	2.01	0.32	6.70	5.9	5.7
133	8699	4	3	4	P3	38.	18.	16.	3.41	1.61	0.32	5.60	5.9	5.7
134	8699	4	3	4	GSG	32.	30.	1.	3.81	0.31	0.32	6.70	5.9	5.7
135	8699	4	3	4	PBS	10.	1.	1.	0.11	0.51	0.32	6.00	5.9	5.7
136	6101	4	3	4	P1	29.	23.	1.	2.01	0.11	1.44	7.10	9.8	8.6
137	6101	4	3	4	P2	42.	27.	22.	2.51	1.21	1.44	9.00	9.8	8.6
138	6101	4	3	4	P3	27.	23.	19.	3.81	2.61	1.44	7.20	9.8	8.6
139	6101	4	3	4	GSG	35.	34.	29.	5.21	3.01	1.44	8.00	9.8	8.6
140	6101	4	3	4	PBS	1.	13.	1.	0.61	-0.59	1.44	7.40	9.8	8.6
141	869N	4	3	4	P1	34.	27.	23.	3.71	1.81	0.28	6.00	5.7	5.4
142	869N	4	3	4	P2	30.	30.	25.	4.01	2.71	0.28	6.80	5.7	5.4
143	869N	4	3	4	P3	31.	30.	24.	4.51	2.41	0.28	6.70	5.7	5.4
144	869N	4	3	4	GSG	35.	36.	35.	6.51	6.21	0.28	7.00	5.7	5.4
145	869N	4	3	4	PBS	1.	1.	1.	-0.99	-0.59	0.28	8.00	5.7	5.4
146	8660	3	2	4	P1	24.	20.	15.	2.11	1.41	3.96	5.10	5.9	5.8
147	8660	3	2	4	P2	33.	17.	14.	1.51	1.21	3.96	6.00	5.9	5.8
148	8660	3	2	4	P3	29.	25.	21.	1.81	2.11	3.96	6.40	5.9	5.8
149	8660	3	2	4	GSG	27.	31.	15.	4.11	1.71	3.96	5.40	5.9	5.8
150	8660	3	2	4	PBS	1.	13.	1.	0.91	-0.39	3.96	6.40	5.9	5.8
151	6103	3	0	5	P1	10.	14.	17.	0.81	1.41	6.70	6.20	3.8	4.7
152	6103	3	0	5	P2	20.	11.	1.	1.01	0.31	6.70	7.00	3.8	4.7

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CASE No.	ANIMAL	TEST	EXPOSURES TO TICKS	STATUS	ANTIGEN	OEDEMA 30 min	ERYTHEMA 4h	ERYTHEMA 24h	4H INC. THICKNESS	24H INC. THICKNESS	% SUSCEPT TO TICKS	NORMALSKIN THICKNESS	END POINT TITRE A	END POINT TITRE B
153	6103	3	0	5	P3	22.	14.	1.	1.41	0.31	6.70	8.00	3.8	4.7
154	6103	3	0	5	GSG	15.	12.	16.	1.01	0.61	6.70	6.30	3.8	4.7
155	6103	3	0	5	PBS	1.	1.	1.	0.81	0.21	6.70	6.40	3.8	4.7
156	8662	2	1	4	P1	40.	34.	21.	1.81	2.11	0.54	7.30	6.2	13.1
157	8662	2	1	4	P2	44.	28.	28.	4.21	5.21	0.54	7.80	6.2	13.1
158	8662	2	1	4	P3	35.	20.	30.	2.81	5.01	0.54	6.70	6.2	13.1
159	8662	2	1	4	GSG	53.	39.	35.	4.61	4.41	0.54	7.60	6.2	13.1
160	8662	2	1	4	PBS	18.	1.	1.	0.51	0.51	0.54	6.80	6.2	13.1
161	8701	1	0	4	P1	8.	1.	13.	2.61	0.91	5.86	6.40	2.7	3.7
162	8701	1	0	4	P2	10.	13.	1.	0.71	0.21	5.86	5.10	2.7	3.7
163	8701	1	0	4	P3	1.	1.	1.	-0.19	0.81	5.86	4.70	2.7	3.7
164	8701	1	0	4	GSG	15.	1.	1.	0.61	0.81	5.86	4.50	2.7	3.7
165	8701	1	0	4	PBS	1.	1.	1.	-0.39	0.41	5.86	5.90	2.7	3.7
166	8676	1	0	4	P1	1.	1.	1.	0.01	0.01	1.92	6.40	2.8	4.5
167	8676	1	0	4	P2	1.	1.	1.	0.81	0.31	1.92	6.70	2.8	4.5
168	8676	1	0	4	P3	1.	1.	1.	0.41	0.01	1.92	6.00	2.8	4.5
169	8676	1	0	4	GSG	28.	23.	24.	1.21	1.11	1.92	6.80	2.8	4.5
170	8676	1	0	4	PBS	1.	1.	1.	1.11	0.21	1.92	8.00	2.8	4.5

DATASET : ACRES2. Chapter seven

ANIMAL	TRAN.SUSC T-1	TRAN.SUSC T-2	TRAN.SUSC T-3	TRAN.SUSC T-4	GROUP	NOR.SKIN THICK T-1	ENDPOINT-A TEST 1	ENDPOINT-B TEST 1	NOR.SKIN THICK T-2	ENDPOINT-A TEST 2	ENDPOINT-B TEST 2	NORM.THICK FINAL-T	ENDPOINT-A FINAL	ENDPOINT B FINAL
8664	1.87	1.25	1.28	0.93	1	4.60	3.10	5.14	4.70	5.14	5.84	8.10	6.81	6.48
8695	2.04	1.43	1.50	1.11	1	4.40	3.18	5.43	5.50	5.43	5.48	7.30	5.40	5.05
8670	1.60	0.85	0.95	0.68	1	4.80	2.75	3.98	4.80	3.98	6.19	6.30	6.72	7.78
8699	1.44	1.17	1.52	0.75	1	4.30	3.62	5.37	4.60	5.37	5.75	6.70	5.90	5.70
6101	1.81	1.45	1.71	1.10	1	5.40	4.27	5.63	5.80	5.63	7.34	8.00	9.82	8.61
869N	1.62	0.84	1.59	0.73	1	5.00	2.17	4.80	4.80	4.80	4.98	7.00	5.69	5.37
8660	2.22	1.68	1.41		2				4.40	3.14	4.84	5.40	5.88	5.84
8662	1.44	0.86			2							6.30	6.21	13.08
8701	1.56				2							7.60	2.67	3.72
8676	1.18				2							4.50	2.84	4.54
6103					3					2.54	2.89	6.80	3.80	4.68
6103					3					2.54	2.89		3.80	4.68
6103	1.61				3					2.54	2.89		3.80	4.68

DATASET : VILLAV.Chapter eight. experiment one

CASE No.	ANIMAL	GROUP	BREED	ANTIGEN	OEDEMA 30'	ERYTHEMA 4 h	ERYTHEMA 24 h	INC. THICKNESS-4 h	INC. THICKNESS- 24H
1	8515	1	H	P1	30	23	1	2.51	1.31
2	8515	1	H	P2	24	1	1	1.61	0.81
3	8515	1	H	P3	24	1	1	1.31	1.21
4	8515	1	H	P4	41	33	1	2.71	1.51
5	8515	1	H	P5	35	23	1	1.31	1.31
6	8515	1	H	P6	62	66	1	4.91	2.31
7	8515	1	H	D2	44	58	1	5.51	2.81
8	8515	1	H	GSG	34	23	1	3.41	2.51
9	8515	1	H	PBS	1	1	1	0.21	1.01
10	8525	1	H	P1	20	19	1	2.01	2.11
11	8525	1	H	P2	20	24	1	1.01	0.91
12	8525	1	H	P3	32	33	1	2.31	1.51
13	8525	1	H	P4	49	56	1	3.21	1.81
14	8525	1	H	P5	15	1	1	1.81	2.41
15	8525	1	H	P6	56	62	1	5.01	1.91
16	8525	1	H	D2	52	52	1	5.11	2.31
17	8525	1	H	GSG	38	36	1	2.91	1.91
18	8525	1	H	PBS	1	1	1	0.21	1.81
19	8531	1	H	P1	17	20	24	1.41	1.41
20	8531	1	H	P2	13	15	20	0.71	1.21
21	8531	1	H	P3	13	14	24	0.71	1.11
22	8531	1	H	P4	29	41	1	3.01	0.91
23	8531	1	H	P5	14	19	1	1.11	1.51
24	8531	1	H	P6	42	43	1	3.81	1.71
25	8531	1	H	D2	23	37	1	4.01	1.61
26	8531	1	H	GSG	28	35	1	3.61	2.51
27	8531	1	H	PBS	12	19	1	2.71	1.11
28	8569	1	H	P1	17	14	1	0.91	0.81
29	8569	1	H	P2	16	21	1	0.01	1.41
30	8569	1	H	P3	24	21	26	1.31	1.41
31	8569	1	H	P4	38	29	1	2.31	0.91
32	8569	1	H	P5	19	19	1	1.31	0.41
33	8569	1	H	P6	38	36	1	3.01	1.51
34	8569	1	H	D2	33	41	1	3.71	0.31
35	8569	1	H	GSG	29	30	1	3.01	1.11
36	8569	1	H	PBS	11	1	1	0.81	0.81
37	8535	1	H	P1	1	1	1	1.31	0.91
38	8535	1	H	P2	26	1	1	0.61	0.11
39	8535	1	H	P3	29	1	1	0.71	0.81
40	8535	1	H	P4	45	36	1	1.71	0.71
41	8535	1	H	P5	30	1	1	1.01	1.01
42	8535	1	H	P6	35	1	1	1.71	0.71
43	8535	1	H	D2	33	34	1	3.01	1.21
44	8535	1	H	GSG	41	1	1	2.41	1.31
45	8535	1	H	PBS	1	1	1	0.21	0.01
46	8567	1	H	P1	22	1	1	1.01	0.41
47	8567	1	H	P2	24	26	1	1.41	1.01
48	8567	1	H	P3	26	1	1	0.81	0.31
49	8567	1	H	P4	38	41	1	2.71	1.31
50	8567	1	H	P5	24	1	1	1.21	0.31

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CASE No.	ANIMAL	GROUP	BREED	ANTIGEN	OEDEMA 30'	ERYTHEMA 4 h	ERYTHEMA 24 h	INC. THICK NESS-4 h	INC. THICK NESS- 24H
51	8567	1	H	P6	38	36	1	2.91	1.21
52	8567	1	H	D2	34	31	1	2.91	1.11
53	8567	1	H	GSG	26	27	1	2.11	0.61
54	8567	1	H	PBS	1	1	1	0.31	0.41
55	8577	1	H	P1	18	1	1	0.81	0.71
56	8577	1	H	P2	16	1	1	1.31	0.71
57	8577	1	H	P3	19	1	1	1.11	0.71
58	8577	1	H	P4	27	1	1	2.01	1.31
59	8577	1	H	P5	20	1	1	1.31	0.81
60	8577	1	H	P6	32	23	1	2.31	0.91
61	8577	1	H	D2	27	27	1	2.11	0.81
62	8577	1	H	GSG	34	1	1	1.81	0.61
63	8577	1	H	PBS	1	1	1	0.61	0.01
64	8539	1	H	P1	27	21	1	1.11	0.41
65	8539	1	H	P2	29	1	1	1.51	0.61
66	8539	1	H	P3	34	1	1	1.61	0.61
67	8539	1	H	P4	36	26	24	2.21	1.31
68	8539	1	H	P5	21	24	1	0.71	0.51
69	8539	1	H	P6	39	34	24	2.91	1.51
70	8539	1	H	D2	35	26	1	1.91	1.11
71	8539	1	H	GSG	28	20	19	3.71	2.01
72	8539	1	H	PBS	1	1	1	0.61	-0.29
73	8563	1	H	P1	19	1	1	0.31	1.11
74	8563	1	H	P2	17	1	16	0.61	0.71
75	8563	1	H	P3	29	1	18	0.71	0.81
76	8563	1	H	P4	28	1	19	1.11	0.91
77	8563	1	H	P5	26	1	1	0.61	0.31
78	8563	1	H	P6	35	29	24	1.41	0.41
79	8563	1	H	D2	34	1	1	1.51	0.61
80	8563	1	H	GSG	40	1	1	1.91	0.11
81	8563	1	H	PBS	1	1	1	0.11	0.01
82	8575	1	H	P1	31	1	1	1.01	0.61
83	8575	1	H	P2	27	1	16	1.71	1.21
84	8575	1	H	P3	31	1	1	1.11	1.11
85	8575	1	H	P4	34	1	1	2.61	1.21
86	8575	1	H	P5	1	30	1	2.41	0.81
87	8575	1	H	P6	42	35	20	3.11	2.01
88	8575	1	H	D2	36	29	23	3.41	0.81
89	8575	1	H	GSG	37	26	1	3.11	2.01
90	8575	1	H	PBS	1	1	1	0.01	0.21
91	8573	1	H	P1	19	1	1	0.91	0.51
92	8573	1	H	P2	21	1	15	1.11	0.91
93	8573	1	H	P3	23	1	1	1.11	0.51
94	8573	1	H	P4	33	1	1	1.71	0.71
95	8573	1	H	P5	31	33	1	1.71	0.21
96	8573	1	H	P6	38	1	1	3.21	1.01
97	8573	1	H	D2	36	31	17	2.81	1.61
98	8573	1	H	GSG	37	1	15	2.51	1.31
99	8573	1	H	PBS	1	1	1	0.51	0.11
100	5347	2	C.SM*HR.PS	P1	23	17	1	1.11	0.71
101	5347	2	C.SM*HR.PS	P2	32	22	1	1.21	0.61

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CASE No.	ANIMAL	GROUP	BREED	ANTIGEN	OEDEMA 30'	ERYTHEMA 4 h	ERYTHEMA 24 h	INC. THICKNESS-4 h	INC. THICKNESS- 24H
102	5347	2	C.SM*HR.PS	P3	32	24	17	1.41	1.01
103	5347	2	C.SM*HR.PS	P4	52	33	16	2.71	1.41
104	5347	2	C.SM*HR.PS	P5	23	18	1	1.41	0.61
105	5347	2	C.SM*HR.PS	P6	29	19	1	2.01	1.01
106	5347	2	C.SM*HR.PS	D2	33	26	21	4.71	1.81
107	5347	2	C.SM*HR.PS	GSG	36	26	15	3.91	1.81
108	5347	2	C.SM*HR.PS	PBS	16	16	14	1.11	0.61
109	6327	2	C.SM*HR.PS	P1	21	21	1	1.41	0.71
110	6327	2	C.SM*HR.PS	P2	23	1	1	0.61	0.61
111	6327	2	C.SM*HR.PS	P3	25	1	1	0.51	0.21
112	6327	2	C.SM*HR.PS	P4	34	30	1	2.41	1.61
113	6327	2	C.SM*HR.PS	P5	22	23	1	2.01	0.91
114	6327	2	C.SM*HR.PS	P6	25	20	1	2.31	0.51
115	6327	2	C.SM*HR.PS	D2	25	19	1	1.31	1.01
116	6327	2	C.SM*HR.PS	GSG	24	15	1	0.81	0.51
117	6327	2	C.SM*HR.PS	PBS	14	1	1	-0.09	-0.39
118	6395	2	M*PS.C	P1	18	1	1	2.01	0.91
119	6395	2	M*PS.C	P2	16	1	1	0.51	0.31
120	6395	2	M*PS.C	P3	17	17	1	0.01	-0.29
121	6395	2	M*PS.C	P4	35	23	1	1.81	1.11
122	6395	2	M*PS.C	P5	25	1	1	1.41	0.61
123	6395	2	M*PS.C	P6	30	22	1	2.61	1.31
124	6395	2	M*PS.C	D2	32	25	1	2.81	2.01
125	6395	2	M*PS.C	GSG	29	23	1	3.11	1.51
126	6395	2	M*PS.C	PBS	1	1	1	0.61	0.01
127	6323	2	J*C	P1	32	26	18	1.21	1.61
128	6323	2	J*C	P2	29	25	1	1.41	1.01
129	6323	2	J*C	P3	27	24	1	1.51	0.61
130	6323	2	J*C	P4	38	33	29	3.71	1.71
131	6323	2	J*C	P5	26	22	1	1.21	0.31
132	6323	2	J*C	P6	40	40	22	4.81	2.11
133	6323	2	J*C	D2	26	29	20	3.81	1.01
134	6323	2	J*C	GSG	35	25	20	3.31	2.11
135	6323	2	J*C	PBS	1	1	1	0.71	-0.19
136	6337	2	M*PS.C	P1	18	1	14	1.21	1.11
137	6337	2	M*PS.C	P2	19	1	1	0.11	0.41
138	6337	2	M*PS.C	P3	26	27	1	0.81	0.61
139	6337	2	M*PS.C	P4	26	27	1	1.91	1.01
140	6337	2	M*PS.C	P5	19	18	1	1.61	0.31
141	6337	2	M*PS.C	P6	29	26	13	2.81	1.21
142	6337	2	M*PS.C	D2	24	19	1	2.51	1.11
143	6337	2	M*PS.C	GSG	27	25	1	3.51	1.71
144	6337	2	M*PS.C	PBS	1	1	1	0.41	0.21
145	6333	2	BON.J	P1	19	20	1	0.01	1.51
146	6333	2	BON.J	P2	16	1	1	0.01	0.11
147	6333	2	BON.J	P3	13	1	1	0.31	0.41
148	6333	2	BON.J	P4	34	28	1	2.81	1.81
149	6333	2	BON.J	P5	21	22	1	0.61	0.31
150	6333	2	BON.J	P6	30	30	23	2.11	0.61
151	6333	2	BON.J	D2	40	27	22	2.31	1.91
152	6333	2	BON.J	GSG	31	21	1	2.71	0.71

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CASE No.	ANIMAL	GROUP	BREED	ANTIGEN	OEDEMA 30'	ERYTHEMA 4 h	ERYTHEMA 24 h	INC. THICKNESS-4 h	INC. THICKNESS- 24H
153	6333	2	BOM.J	PBS	1	1	1	0.71	0.61
154	6349	2	HR.C	P1	18	20	1	0.61	0.11
155	6349	2	HR.C	P2	16	1	1	0.11	0.31
156	6349	2	HR.C	P3	20	25	1	0.81	0.71
157	6349	2	HR.C	P4	27	29	1	2.51	1.41
158	6349	2	HR.C	P5	22	16	1	1.01	0.41
159	6349	2	HR.C	P6	25	32	1	2.61	1.11
160	6349	2	HR.C	D2	24	28	1	2.31	1.01
161	6349	2	HR.C	GSG	29	30	1	2.81	1.31
162	6349	2	HR.C	PBS	1	1	1	-0.69	-0.49
163	8519	1	H	P1	18	16	1	0.51	0.31
164	8519	1	H	P2	18	17	15	0.21	0.41
165	8519	1	H	P3	22	17	1	1.21	1.11
166	8519	1	H	P4	23	23	15	1.91	0.51
167	8519	1	H	P5	15	15	1	1.01	0.41
168	8519	1	H	P6	30	23	20	2.51	1.21
169	8519	1	H	D2	28	27	16	2.81	0.91
170	8519	1	H	GSG	24	21	1	1.61	1.01
171	8519	1	H	PBS	10	14	1	1.01	0.11
172	8597	1	H	P1	29	1	1	1.31	0.91
173	8597	1	H	P2	30	25	1	1.41	0.61
174	8597	1	H	P3	25	22	1	1.21	0.31
175	8597	1	H	P4	1	49	1	3.11	1.21
176	8597	1	H	P5	33	29	1	1.41	0.41
177	8597	1	H	P6	31	47	1	2.81	0.51
178	8597	1	H	D2	32	43	14	3.71	1.31
179	8597	1	H	GSG	33	25	1	2.91	0.81
180	8597	1	H	PBS	10	12	1	1.81	0.51
181	8563	1	H	P1	19	1	1	0.31	0.21
182	8563	1	H	P2	17	1	1	0.61	0.71
183	8563	1	H	P3	23	1	1	0.41	0.71
184	8563	1	H	P4	37	21	1	1.71	0.81
185	8563	1	H	P5	18	20	1	0.51	0.11
186	8563	1	H	P6	31	26	1	1.71	0.21
187	8563	1	H	D2	28	26	1	2.01	0.21
188	8563	1	H	GSG	29	26	1	2.61	0.81
189	8563	1	H	PBS	10	19	1	1.91	0.21
190	8545	1	H	P1	20	18	1	1.01	0.61
191	8545	1	H	P2	19	18	1	1.31	0.41
192	8545	1	H	P3	21	21	1	1.21	0.41
193	8545	1	H	P4	25	28	1	2.41	1.11
194	8545	1	H	P5	15	17	1	1.41	0.11
195	8545	1	H	P6	26	28	21	4.71	2.21
196	8545	1	H	D2	22	25	15	4.31	1.81
197	8545	1	H	GSG	22	25	20	4.51	1.31
198	8545	1	H	PBS	1	16	1	1.71	0.51
199	6351	2	PS(HR.C)	P1	14	1	1	1.11	0.81
200	6351	2	PS(HR.C)	P2	19	19	1	1.11	-0.19
201	6351	2	PS(HR.C)	P3	20	19	1	0.81	0.91
202	6351	2	PS(HR.C)	P4	31	18	1	1.61	1.11
203	6351	2	PS(HR.C)	P5	21	16	1	1.21	0.01

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CASE No.	ANIMAL	GROUP	BREED	ANTIGEN	OEDEMA 30'	ERYTHEMA 4 h	ERYTHEMA 24 h	INC. THICKNESS-4 h	INC. THICKNESS- 24H
204	6351	2	PS(HR.C)	P6	37	27	1	2.71	0.31
205	6351	2	PS(HR.C)	D2	22	24	1	2.81	0.11
206	6351	2	PS(HR.C)	GSG	27	26	1	3.01	0.51
207	6351	2	PS(HR.C)	PBS	1	11	1	1.21	0.01
208	6313	2	C.SM*HR.PS	P1	27	23	1	0.91	0.01
209	6313	2	C.SM*HR.PS	P2	24	18	1	1.11	0.21
210	6313	2	C.SM*HR.PS	P3	26	26	1	1.61	0.81
211	6313	2	C.SM*HR.PS	P4	37	50	17	3.01	1.61
212	6313	2	C.SM*HR.PS	P5	21	30	1	1.81	0.41
213	6313	2	C.SM*HR.PS	P6	28	42	1	2.81	0.91
214	6313	2	C.SM*HR.PS	D2	34	32	22	2.51	1.71
215	6313	2	C.SM*HR.PS	GSG	20	29	23	3.31	1.31
216	6313	2	C.SM*HR.PS	PBS	11	20	1	1.91	0.31
217	6343	2	M(PS.C)	P1	16	14	1	1.31	0.31
218	6343	2	M(PS.C)	P2	15	15	1	1.11	0.61
219	6343	2	M(PS.C)	P3	23	1	1	1.11	0.61
220	6343	2	M(PS.C)	P4	32	25	1	3.31	1.31
221	6343	2	M(PS.C)	P5	18	18	1	1.71	0.51
222	6343	2	M(PS.C)	P6	27	24	1	3.81	1.21
223	6343	2	M(PS.C)	D2	21	35	1	5.41	1.61
224	6343	2	M(PS.C)	GSG	21	25	1	3.41	1.11
225	6343	2	M(PS.C)	PBS	1	14	1	1.11	0.01
226	8557	1	H	P1	19	1	1	1.11	0.51
227	8557	1	H	P2	31	1	1	1.51	0.21
228	8557	1	H	P3	35	29	1	2.51	0.41
229	8557	1	H	P4	42	40	1	3.31	0.51
230	8557	1	H	P5	35	21	1	1.81	-0.19
231	8557	1	H	P6	47	46	1	5.21	1.21
232	8557	1	H	D2	30	25	1	3.71	1.61
233	8557	1	H	GSG	33	32	1	4.01	1.21
234	8557	1	H	PBS	1	18	1	2.31	0.31
235	8739	3	H	P1	1	1	1	0.31	0.11
236	8739	3	H	P2	1	1	1	0.71	0.41
237	8739	3	H	P3	1	1	1	0.91	0.51
238	8739	3	H	P4	1	1	1	1.21	0.61
239	8739	3	H	P5	1	1	1	1.01	0.61
240	8739	3	H	P6	1	11	12	0.91	0.51
241	8739	3	H	D2	1	1	1	1.71	0.51
242	8739	3	H	GSG	1	1	1	0.11	0.21
243	8739	3	H	PBS	1	1	1	0.71	0.01
244	1010	3	H	P1	11	10	11	0.61	0.71
245	1010	3	H	P2	1	1	1	0.31	0.51
246	1010	3	H	P3	1	1	1	0.51	0.51
247	1010	3	H	P4	1	16	14	0.91	1.11
248	1010	3	H	P5	13	19	10	1.41	0.71
249	1010	3	H	P6	1	1	1	0.51	0.21
250	1010	3	H	D2	15	18	1	0.91	0.91
251	1010	3	H	GSG	1	1	1	0.61	0.41
252	1010	3	H	PBS	1	1	1	0.51	0.21

DATASET : VILLAV.Chapter eight. experiment one

ANIMAL	GROUP	BREED	TOT.FIELD COUNT	LOG MEAN FIELD CNT	TOT.TICKS ART INFEST	% SUSCEP TO TICKS	DATES FILD COUNT	DATES ARTF TICK CONT	DATES SKIN TEST	NORMAL SKIN THICKNES
8515	1	H	299	1.86	197	3.94	25/ 7/87	20/ 5/87	2/ 9/87	6.80
8525	1	H	521	2.06	161	3.22	25/ 7/87	20/ 5/87	2/ 9/87	6.80
8531	1	H	447	2.00	303	6.06	25/ 7/87	20/ 5/87	2/ 9/87	5.00
8569	1	H	328	1.90	212	4.24	25/ 7/87	20/ 5/87	2/ 9/87	4.00
8535	1	H	865	2.17	313	6.26	25/ 7/87	20/ 5/87	7/ 9/87	4.20
8567	1	H	1268	2.39	447	8.94	25/ 7/87	20/ 5/87	7/ 9/87	3.70
8577	1	H	421	1.97	530	10.60	25/ 7/87	20/ 5/87	7/ 9/87	3.20
8539	1	H	243	1.77	76	1.52	25/ 7/87	20/ 5/87	7/ 9/87	5.20
8563	1	H	445	2.02	203	4.06	25/ 7/87	20/ 5/87	7/ 9/87	3.90
8575	1	H	485	1.99	176	3.52	25/ 7/87	20/ 5/87	7/ 9/87	4.70
8573	1	H	325	1.85	141	2.82	25/ 7/87	20/ 5/87	7/ 9/87	3.40
5347	2	C.SM*HR.PS	672	2.07	219	4.38	14/ 9/87	5/11/87	8/ 9/87	6.40
6327	2	C.SM*HR.PS	2399	2.73	237	4.74	14/ 9/87	5/11/87	8/ 9/87	8.70
6395	2	M*PS.C	990	2.37	363	7.26	14/ 9/87	5/11/87	8/ 9/87	7.70
6323	2	J*C	706	2.13	224	4.48	14/ 9/87	5/11/87	8/ 9/87	6.50
6337	2	M*PS.C	909	2.31	282	5.64	14/ 9/87	5/11/87	8/ 9/87	4.10
6333	2	BON.J	1104	2.42	454	9.08	14/ 9/87	5/11/87	8/ 9/87	9.00
6349	2	HR.C	897	2.29	308	6.16	14/ 9/87	5/11/87	8/ 9/87	6.30
8519	1	H	327	1.84	135	2.70	25/ 7/87	20/ 5/87	28/ 9/87	5.70
8597	1	H	207	1.70	214	4.28	25/ 7/87	20/ 5/87	28/ 9/87	4.20
8563	1	H	445	2.02	203	4.06	25/ 7/87	20/ 5/87	28/ 9/87	4.00
8545	1	H	417	1.98	165	3.30	25/ 7/87	20/ 5/87	28/ 9/87	5.30
6351	2	PS(HR.C)	757	2.25	302	6.04	14/ 9/87	5/11/87	28/ 9/87	4.90
6313	2	C.SM*HR.PS	927	2.33	300	6.00	14/ 9/87	5/11/87	28/ 9/87	4.10
6343	2	M(PS.C)	934	2.33	364	7.28	14/ 9/87	5/11/87	28/ 9/87	5.10
8557	1	H	696	2.20	397	7.94	25/ 7/87	20/ 5/85	28/ 9/87	5.50
8739	3	H								
1010	3	H								

DATASET : L465E-Chapter eight. experiment two

CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 MINUTES	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	INC.THICKNESS 4H	INC.THICKNESS 24H	DATES SKIN TESTS	NORMAL SKIN THICKNESS
1	6389	P1	1	1	1	2.71	0.31	26/10/87	10.00
2	6389	P3	1	1	1	2.11	0.11	26/10/87	11.20
3	6389	P4	24	19	18	1.41	0.81	26/10/87	10.20
4	6389	P5	1	32	1	2.01	1.41	26/10/87	15.00
5	6389	P6	1	51	1	4.11	2.11	26/10/87	16.90
6	6389	D2	1	1	1	0.21	0.01	26/10/87	10.00
7	6389	GSG	1	42	29	5.51	2.91	26/10/87	13.00
8	6389	PBS	1	1	1	0.31	1.61	26/10/87	13.50
9	6385	P1	1	1	1	0.31	0.21	26/10/87	5.30
10	6385	P3	1	1	1	1.21	0.61	26/10/87	6.50
11	6385	P4	25	21	18	1.11	1.21	26/10/87	5.90
12	6385	P5	22	28	17	4.01	1.31	26/10/87	6.00
13	6385	P6	21	15	1	1.91	0.71	26/10/87	6.00
14	6385	D2						26/10/87	6.00
15	6385	GSG	26	27	25	2.71	1.81	26/10/87	6.20
16	6385	PBS	1	1	1	0.21	0.11	26/10/87	6.00
17	6375	P1	1	1	1	0.41	0.21	26/10/87	5.60
18	6375	P3	21	1	1	0.61	0.51	26/10/87	6.80
19	6375	P4	32	25	19	2.71	1.21	26/10/87	7.00
20	6375	P5	35	20	1	2.31	1.21	26/10/87	5.70
21	6375	P6	20	22	16	2.51	1.01	26/10/87	6.50
22	6375	D2	26	25	1	1.91	1.01	26/10/87	7.50
23	6375	GSG	24	23	17	2.11	1.11	26/10/87	6.40
24	6375	PBS	1	1	1	0.71	0.61	26/10/87	6.20
25	7301	P1	1	13	1	0.41	0.21	26/10/87	4.60
26	7301	P3	19	1	1	0.91	0.41	26/10/87	4.90
27	7301	P4	31	18	15	1.61	0.91	26/10/87	5.70
28	7301	P5	31	20	1	1.91	0.71	26/10/87	4.80
29	7301	P6	21	18	16	1.41	1.21	26/10/87	5.80
30	7301	D2	16	11	1	1.31	0.21	26/10/87	4.50
31	7301	GSG	23	21	18	2.91	2.21	26/10/87	5.10
32	7301	PBS	1	1	1	0.11	0.01	26/10/87	5.40
33	6369	P1	22	1	1	0.61	0.21	26/10/87	4.30
34	6369	P3	24	1	1	0.21	0.01	26/10/87	5.00
35	6369	P4	41	15	13	1.61	1.01	26/10/87	4.90
36	6369	P5	33	17	13	1.81	0.71	26/10/87	5.10
37	6369	P6	26	29	1	0.51	0.11	26/10/87	5.20
38	6369	D2	32	25	15	0.81	0.11	26/10/87	5.20
39	6369	GSG	45	1	16	0.71	1.41	26/10/87	5.30
40	6369	PBS	1	1	1	0.31	0.21	26/10/87	4.70
41	6367	P1	11	1	1	0.51	0.01	26/10/87	3.70
42	6367	P3	27	1	1	1.01	0.61	26/10/87	4.10
43	6367	P4	38	20	13	2.11	0.81	26/10/87	4.20
44	6367	P5	47	27	1	2.31	0.91	26/10/87	3.80
45	6367	P6	38	1	11	1.61	0.81	26/10/87	4.20
46	6367	D2	26	1	1	1.31	0.11	26/10/87	4.70
47	6367	GSG	38	26	17	2.71	2.81	26/10/87	4.30
48	6367	PBS	1	1	1	0.51	0.91	26/10/87	4.70
49	6321	P1	17	1	1	0.61	0.31	26/10/87	6.90
50	6321	P3	18	1	1	0.41	0.01	26/10/87	6.60

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 MINUTES	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	INC.THICKNESS 4H	INC.THICKNESS 24H	DATES SKIN TESTS	NORMAL SKIN THICKNESS
51	6321	P4						26/10/87	
52	6321	P5	47	25	15	3.51	1.11	26/10/87	7.60
53	6321	P6	26	22	1	2.61	0.21	26/10/87	5.80
54	6321	D2	37	19	1	2.41	0.41	26/10/87	6.70
55	6321	GSG	38	34	1	3.01	1.31	26/10/87	7.10
56	6321	PBS	1	1	1	0.11	0.31	26/10/87	6.50
57	8597	P1	30	18	1	1.11	0.61	9/11/87	5.10
58	8597	P3	47	23	14	1.81	0.91	9/11/87	4.90
59	8597	P4	59	32	26	2.31	1.61	9/11/87	5.00
60	8597	P5	37	26	18	1.71	0.51	9/11/87	5.20
61	8597	P6	43	32	1	1.71	0.51	9/11/87	5.30
62	8597	D2	32	27	24	2.51	1.31	9/11/87	5.40
63	8597	GSG	31	29	27	2.81	2.01	9/11/87	4.70
64	8597	PBS	1	1	1	1.01	0.61	9/11/87	4.80
65	8563	P1	12	1	1	0.81	1.21	9/11/87	3.30
66	8563	P3	22	1	1	1.01	0.71	9/11/87	3.80
67	8563	P4	32	22	23	1.61	1.71	9/11/87	3.70
68	8563	P5	29	1	1	0.61	1.01	9/11/87	5.20
69	8563	P6	35	26	1	1.31	0.91	9/11/87	4.20
70	8563	D2	30	26	1	1.71	0.61	9/11/87	4.10
71	8563	GSG	35	26	24	2.11	1.01	9/11/87	4.20
72	8563	PBS	1	1	1	1.41	0.41	9/11/87	3.80
73	8573	P1	13	1	1	0.71	0.61	9/11/87	3.50
74	8573	P3	28	1	1	1.31	0.41	9/11/87	3.80
75	8573	P4	37	31	17	2.51	1.21	9/11/87	3.90
76	8573	P5	19	29	1	1.51	0.21	9/11/87	4.00
77	8573	P6	34	31	1	2.21	0.51	9/11/87	4.20
78	8573	D2	34	42	17	2.11	0.61	9/11/87	4.30
79	8573	GSG	27	33	21	2.91	0.91	9/11/87	4.30
80	8573	PBS	1	19	1	0.21	0.21	9/11/87	4.20
81	8531	P1	21	19	1	1.01	0.91	9/11/87	5.20
82	8531	P3	31	27	24	1.51	0.81	9/11/87	5.80
83	8531	P4	41	31	26	1.81	0.61	9/11/87	5.50
84	8531	P5	20	23	1	1.51	0.41	9/11/87	5.80
85	8531	P6	32	39	1	3.61	1.11	9/11/87	5.40
86	8531	D2	24	27	20	3.21	1.41	9/11/87	5.20
87	8531	GSG	29	31	22	4.51	1.41	9/11/87	5.60
88	8531	PBS	1	1	1	0.01	0.31	9/11/87	6.40
89	8575	P1	17	18	20	1.71	1.61	9/11/87	4.30
90	8575	P3	28	23	20	1.61	1.41	9/11/87	5.00
91	8575	P4	45	33	20	2.51	1.11	9/11/87	5.30
92	8575	P5	52	34	31	4.21	2.81	9/11/87	4.80
93	8575	P6	40	37	22	3.41	1.41	9/11/87	5.10
94	8575	D2	33	35	22	3.21	1.21	9/11/87	5.30
95	8575	GSG						9/11/87	5.40
96	8575	PBS	1	19	1	0.91	0.11	9/11/87	5.10
97	8535	P1	14	1	1	1.31	0.91	9/11/87	4.00
98	8535	P3	28	27	1	1.31	0.51	9/11/87	4.50
99	8535	P4	27	29	19	1.61	0.91	9/11/87	4.80
100	8535	P5	28	23	26	2.71	1.81	9/11/87	4.60
101	8535	P6	29	23	18	2.51	1.21	9/11/87	5.00

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 MINUTES	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	INC.THICKNESS 4H	INC.THICKNESS 24H	DATES SKIN TESTS	NORMAL SKIN THICKNESS
102	8535	D2	25	24	1	3.11	1.21	9/11/87	4.30
103	8535	GSG	24	33	24	2.91	2.01	9/11/87	4.70
104	8535	PBS	1	1	1	0.81	0.51	9/11/87	4.90
105	8539	P1	20	28	1	0.81	0.91	9/11/87	5.80
106	8539	P3	36	30	1	2.11	0.81	9/11/87	5.60
107	8539	P4	40	56	41	3.21	1.61	9/11/87	6.20
108	8539	P5	49	59	31	4.71	2.41	9/11/87	5.80
109	8539	P6	31	38	24	3.11	1.61	9/11/87	5.90
110	8539	D2	39	32	22	1.91	0.71	9/11/87	5.50
111	8539	GSG	42	44	31	5.31	2.01	9/11/87	5.50
112	8539	PBS	1	23	1	1.31	0.01	9/11/87	5.50
113	8569	P1	35	36	1	1.61	0.41	10/12/87	5.50
114	8569	P3						10/12/87	
115	8569	P4	41	47	17	2.21	0.61	10/12/87	5.00
116	8569	P5	41	45	1	2.71	0.51	10/12/87	5.00
117	8569	P6	38	35	18	2.71	2.11	10/12/87	5.60
118	8569	D2	40	37	1	3.11	0.51	10/12/87	5.30
119	8569	GSG	27	34	21	3.81	1.31	10/12/87	5.40
120	8569	PBS	1	16	1	1.21	-0.09	10/12/87	5.50
121	8549	P1	24	25	1	1.41	0.61	10/12/87	3.20
122	8549	P3						10/12/87	
123	8549	P4	46	47	1	1.71	0.51	10/12/87	3.80
124	8549	P5	39	39	1	3.41	0.01	10/12/87	3.40
125	8549	P6	33	29	1	1.61	0.11	10/12/87	3.40
126	8549	D2	26	26	1	1.51	-0.09	10/12/87	3.90
127	8549	GSG	22	29	19	1.91	1.01	10/12/87	3.80
128	8549	PBS	1	1	1	0.81	0.41	10/12/87	3.60
129	8585	P1	36	46	1	1.61	-0.09	10/12/87	3.90
130	8585	P3						10/12/87	
131	8585	P4	45	50	1	2.01	1.11	10/12/87	4.00
132	8585	P5	47	66	14	2.31	1.41	10/12/87	3.90
133	8585	P6	38	40	1	2.31	0.31	10/12/87	4.40
134	8585	D2	36	52	16	2.61	1.31	10/12/87	4.00
135	8585	GSG	33	39	16	4.01	1.51	10/12/87	4.00
136	8585	PBS	1	1	1	0.01	-0.69	10/12/87	4.00
137	8545	P1	35	43	1	1.51	-0.49	10/12/87	5.50
138	8545	P3						10/12/87	
139	8545	P4	33	41	16	2.01	0.31	10/12/87	6.00
140	8545	P5	22	38	1	1.61	0.21	10/12/87	6.00
141	8545	P6	36	53	1	0.91	-0.59	10/12/87	6.60
142	8545	D2	27	34	20	2.81	0.61	10/12/87	6.00
143	8545	GSG	25	33	20	3.01	0.91	10/12/87	5.40
144	8545	PBS	1	12	1	0.61	0.41	10/12/87	5.70
145	8525	P1	36	41	1	1.41	-0.09	10/12/87	6.60
146	8525	P3						10/12/87	
147	8525	P4	50	52	20	3.61	1.41	10/12/87	7.40
148	8525	P5	43	36	23	2.81	1.01	10/12/87	7.70
149	8525	P6	46	44	26	3.81	0.51	10/12/87	8.00
150	8525	D2	52	42	22	3.31	1.01	10/12/87	7.60
151	8525	GSG	46	41	21	4.51	1.71	10/12/87	7.80
152	8525	PBS	1	1	1	0.71	-0.29	10/12/87	7.60

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 MINUTES	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	INC.THICKNESS 4H	INC.THICKNESS 24H	DATES SKIN TESTS	NORMAL SKIN THICKNESS
153	8515	P1	43	51	1	1.91	0.51	10/12/87	6.30
154	8515	P3						10/12/87	
155	8515	P4	75	74	20	5.51	1.31	10/12/87	6.50
156	8515	P5	67	71	1	5.11	0.51	10/12/87	7.00
157	8515	P6	53	51	19	3.41	0.51	10/12/87	7.10
158	8515	D2	66	65	22	4.71	1.01	10/12/87	7.00
159	8515	GSG	53	48	21	4.41	1.61	10/12/87	6.40
160	8515	PBS	1	1	1	2.01	-0.09	10/12/87	7.00
161	8557	P1	42	42	1	2.91	0.61	10/12/87	6.00
162	8557	P3						10/12/87	
163	8557	P4	56	48	20	2.51	0.11	10/12/87	6.60
164	8557	P5	71	68	26	4.81	1.11	10/12/87	6.50
165	8557	P6	52	57	21	3.21	1.01	10/12/87	6.00
166	8557	D2	54	50	21	4.21	1.41	10/12/87	6.30
167	8557	GSG	49	51	29	4.21	1.91	10/12/87	6.80
168	8557	PBS	1	1	1	0.61	-0.09	10/12/87	6.80
169	8400	P1	36	35	1	2.01	0.41	10/12/87	6.00
170	8400	P3						10/12/87	
171	8400	P4	32	38	21	2.21	1.61	10/12/87	6.50
172	8400	P5	34	39	24	2.51	1.41	10/12/87	5.90
173	8400	P6	32	33	1	2.31	0.81	10/12/87	7.00
174	8400	D2	29	32	1	2.01	0.51	10/12/87	6.70
175	8400	GSG	30	38	25	3.81	1.41	10/12/87	7.20
176	8400	PBS	1	19	1	1.31	0.31	10/12/87	6.70
177	5397	P1	14	18	1	-0.19	-0.19	23/11/87	8.20
178	5397	P3	26	20	1	2.81	1.61	23/11/87	8.40
179	5397	P4	32	28	23	2.11	1.31	23/11/87	8.80
180	5397	P5	45	31	31	3.61	2.21	23/11/87	9.20
181	5397	P6	40	30	31	1.81	1.21	23/11/87	10.40
182	5397	D2	25	26	1	3.11	1.21	23/11/87	7.80
183	5397	GSG	41	30	28	5.11	2.01	23/11/87	8.80
184	5397	PBS	1	1	1	0.61	-1.49	23/11/87	8.90
185	5385	P1	20	17	1	1.41	0.51	23/11/87	7.30
186	5385	P3	27	20	1	1.91	0.61	23/11/87	7.70
187	5385	P4	48	37	22	2.31	1.31	23/11/87	7.80
188	5385	P5	43	30	27	3.91	1.71	23/11/87	7.70
189	5385	P6	42	30	1	3.11	0.91	23/11/87	7.80
190	5385	D2	38	27	1	3.01	1.21	23/11/87	8.00
191	5385	GSG	44	28	20	3.51	1.41	23/11/87	7.00
192	5385	PBS	14	1	1	0.21	-0.19	23/11/87	7.50
193	5321	P1	36	1	1	1.31	0.91	23/11/87	7.30
194	5321	P3	51	1	1	1.31	0.81	23/11/87	7.70
195	5321	P4	60	26	1	2.41	1.21	23/11/87	7.50
196	5321	P5	67	41	29	3.51	2.51	23/11/87	8.00
197	5321	P6	59	31	36	2.21	1.51	23/11/87	9.00
198	5321	D2	45	26	25	2.11	1.01	23/11/87	7.60
199	5321	GSG	66	39	29	2.61	3.11	23/11/87	7.90
200	5321	PBS	1	1	1	0.61	0.01	23/11/87	8.50
201	5379	P1	18	1	1	2.11	0.41	23/11/87	7.10
202	5379	P3	29	22	1	2.91	1.81	23/11/87	6.10
203	5379	P4	41	1	1	1.11	0.31	23/11/87	5.90

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 MINUTES	ERYTHEMA 4 HOURS	ERYTHEMA 24 HOURS	INC.THICKNESS 4H	INC.THICKNESS 24H	DATES SKIN TESTS	NORMAL SKIN THICKNESS
204	5379	P5	44	1	18	1.41	0.01	23/11/87	7.00
205	5379	P6	36	25	20	2.81	1.71	23/11/87	5.90
206	5379	D2	33	24	1	1.71	0.61	23/11/87	6.00
207	5379	GSG	39	29	25	3.01	0.91	23/11/87	6.40
208	5379	PBS	1	1	1	0.81	0.01	23/11/87	6.70
209	6511	P1	18	17	16	1.61	0.51	23/11/87	7.00
210	6511	P3	27	19	21	2.61	1.51	23/11/87	6.70
211	6511	P4	50	24	25	3.21	2.01	23/11/87	7.50
212	6511	P5	53	30	35	4.21	1.61	23/11/87	7.90
213	6511	P6	56	29	30	3.61	1.91	23/11/87	8.40
214	6511	D2	59	39	42	3.81	1.91	23/11/87	8.60
215	6511	GSG	42	28	22	3.81	1.81	23/11/87	7.60
216	6511	PBS	1	1	1	0.61	-0.29	23/11/87	8.50
217	5381	P1	14	21	1	2.21	-0.29	23/11/87	10.60
218	5381	P3	14	24	21	3.51	2.91	23/11/87	10.00
219	5381	P4	39	39	1	1.51	0.01	23/11/87	13.00
220	5381	P5	54	40	34	3.41	2.01	23/11/87	12.50
221	5381	P6	37	36	1	2.51	1.81	23/11/87	12.60
222	5381	D2	30	1	1	1.21	-0.59	23/11/87	12.20
223	5381	GSG	45	39	31	4.61	1.51	23/11/87	12.10
224	5381	PBS	1	1	1	-0.09	-0.79	23/11/87	
225	5405	P1	28	1	1	0.91	0.31	23/11/87	9.60
226	5405	P3	42	1	1	2.81	2.71	23/11/87	8.70
227	5405	P4	55	38	36	4.51	2.21	23/11/87	9.50
228	5405	P5	54	47	32	10.21	3.81	23/11/87	9.60
229	5405	P6	53	38	40	5.41	1.91	23/11/87	11.00
230	5405	D2	57	47	29	5.41	2.41	23/11/87	9.30
231	5405	GSG	42	38	33	5.31	1.71	23/11/87	10.30
232	5405	PBS	1	1	1	0.21	0.11	23/11/87	10.40
233	5325	P1	17		1		1.01	23/11/87	108.00
234	5325	P3	23		1		0.41	23/11/87	9.60
235	5325	P4	49		1		1.51	23/11/87	9.20
236	5325	P5	38		29		1.31	23/11/87	11.30
237	5325	P6	33		1		0.91	23/11/87	10.70
238	5325	D2	47		37		-3.49	23/11/87	10.80
239	5325	GSG	49		26		1.31	23/11/87	9.50
240	5325	PBS	1		1		1.01	23/11/87	10.00
241	5371	P1	21		1		2.21	23/11/87	8.50
242	5371	P3	26		22		0.81	23/11/87	9.20
243	5371	P4	34		27		1.81	23/11/87	9.20
244	5371	P5	28		27		2.71	23/11/87	9.10
245	5371	P6	35		27		1.81	23/11/87	10.20
246	5371	D2	30		26		0.91	23/11/87	10.30
247	5371	GSG	37		30		2.71	23/11/87	10.30
248	5371	PBS	11		1		1.31	23/11/87	10.20

DATASET : L465E-Chapter eight. experiment two

CASE No.	ANIMAL	GROUP	BREED	LOG. MEAN FIELD COUN	TOTAL TICK S ART.INFS	SUSCEPTIB. TO TICKS	DATES ARTI F. TICK C.	DATES FIEL D T. COUNT
7	6389	2	SM.C*PS.SM	1.68	87	1.74	21/12/87	1/11/87
15	6385	2	PS*C	1.69	177	3.54	21/12/87	1/11/87
23	6375	2	C*SM	1.54	228	4.56	21/12/87	1/11/87
31	7301	2	HR*C	1.45	179	3.58	21/12/87	1/11/87
39	6369	2	HR*C	1.82	298	5.96	21/12/87	1/11/87
47	6367	2	HR.C*M.C	1.70	264	5.28	21/12/87	1/11/87
55	6321	2	J	2.14	313	6.26	21/12/87	1/11/87
63	8597	1	H	0.84	298	5.96	9/ 2/88	16/12/87
71	8563	1	H	1.26	1150	23.00	9/ 2/88	16/12/87
79	8573	1	H	0.81	756	15.12	9/ 2/88	16/12/87
87	8531	1	H	0.66	537	10.74	9/ 2/88	16/12/87
95	8575	1	H	1.03	660	13.20	9/ 2/88	16/12/87
103	8535	1	H	1.42	1015	20.30	9/ 2/88	16/12/87
111	8539	1	H	0.98	713	14.26	9/ 2/88	16/12/87
119	8569	1	H	1.29	698	13.96	9/ 2/88	16/12/87
127	8549	1	H	1.40	1442	28.84	9/ 2/88	16/12/87
135	8585	1	H	1.23	773	15.46	9/ 2/88	16/12/87
143	8545	1	H	1.45	742	14.84	9/ 2/88	16/12/87
151	8525	1	H	1.05	867	17.34	9/ 2/88	16/12/87
159	8515	1	H	0.20			9/ 2/88	16/12/87
167	8557	1	H	0,96	1434	28.68	9/ 2/88	16/12/87
175	8400	1	H	1.06	1012	20.24	9/ 2/88	16/12/87
183	5397	2	PS*C.SM		60	1.20	21/12/87	
191	5385	2	PS*C		143	2.86	21/12/87	
199	5321	2	PS*HR.C		227	4.54	21/12/87	
207	5379	2	M*PS.C		85	1.70	21/12/87	
215	6511	2	SM*BON.J		87	1.74	21/12/87	
223	5381	2	SM*C		117	2.34	21/12/87	
231	5405	2	SM*C		125	2.50	21/12/87	
239	5325	2	J		294	5.88	21/12/87	
247	5371	2	PS*C		135	2.70	21/12/87	

DATASET : ELIADQ. Chapter seven. ELISA

CASE No.	ANIMAL	STATUS	MEAN READ	END POINT TITRE
1	8660	0	310	3.40
2	8662	0	297	3.30
3	8664	0	272	3.10
4	8670	0	231	2.75
5	8676	0	186	2.33
6	8695	0	282	3.18
7	8699	0	339	3.62
8	869N	0	170	2.17
9	6101	0	441	4.27
10	6103	0	152	1.98
11	8701	0	243	2.86
12	8660	0	276	3.14
13	8664	1	638	5.14
14	8670	1	393	3.98
15	8695	1	734	5.43
16	8699	1	711	5.37
17	869N	1	548	4.80
18	6101	1	823	5.63
19	6103	0	208	2.54
20	8660	1	559	4.84
21	8662	0	244	2.87
22	8664	2	935	5.84
23	8670	2	1158	6.19
24	8695	2	755	5.48
25	8699	2	882	5.75
26	869N	2	594	4.98
27	6101	2	1573	7.34
28	6103	1	247	2.89
29	8660	2	958	5.88
30	8662	1	1171	6.21
31	8664	3	1435	6.81
32	8670	3	1404	6.72
33	8676	0	241	2.84
34	8695	3	725	5.40
35	8699	3	973	5.90
36	869N	3	850	5.69
37	6101	3	1925	9.82
38	6103	2	366	3.80
39	8701	0	222	2.67
40	8660	3	936	5.84
41	8662	2	2180	13.08
42	8664	4	1312	6.48

DATASET : DOSRET1. Chapter nine. test one.

CASE No.	ANIMAL	ANTIGEN	OEDEMA 30. min dil1	ERYTHEMA 4 h DIL 1	INC.THICK 4h DIL 1	OEDEMA 30 min DIL 2	ERYTHEA 4 h DIL 2	INC.THICK 4h DIL 2	OEDEMA 30 min DIL 3	ERYTHEMA 4 h DIL 3	INC.THICK 4H DIL 3	TRANS.SUSC EPT TEST 1	ELISA TEST 1
1	8660	P1	22.45	18.49	1.31	16.97	17.89	1.31	1.00	17.32	0.01	0.63	5.68
2	8660	P3	37.42	26.50	2.01	25.50	18.49	1.91	24.49	23.24	1.11	0.63	5.68
3	8660	P4	35.99	24.98	2.71	30.40	23.92	2.21	30.50	20.35	2.01	0.63	5.68
4	8660	P6	28.98	19.44	2.41	1.00	1.00	0.21	22.49	1.00	1.61	0.63	5.68
5	8662	P1	32.00	32.00	4.21	30.46	20.90	2.81	17.44	1.00	0.81	1.09	9.39
6	8662	P3	34.50	21.45	3.41	36.47	23.92	4.41	32.50	23.24	1.51	1.09	9.39
7	8662	P4	39.80	33.47	7.41	35.99	30.94	3.51	34.47	26.50	1.11	1.09	9.39
8	8662	P6	44.96	28.72	5.21	32.94	24.45	2.31	28.46	1.00	1.81	1.09	9.39
9	8664	P1				1.00	1.00	0.81	1.00	1.00	1.31	1.37	3.37
10	8664	P3	37.47	20.49	1.81	30.85	1.00	2.01	17.49	1.00	1.71	1.37	3.37
11	8664	P4	47.37	29.98	3.01	30.46	22.49	1.71	21.00	23.37	1.71	1.37	3.37
12	8664	P6	19.44	18.33	3.21	1.00	15.49	1.41	1.00	1.00	0.11	1.37	3.37
13	8666	P1	29.85	1.00	1.41	27.00	1.00	0.71	21.98	1.00	0.21	1.21	4.29
14	8666	P3	37.42	14.87	1.61	29.46	15.49	0.31	1.00	1.00	0.91	1.21	4.29
15	8666	P4	32.50	18.44	2.21	49.30	19.00	1.21	30.46	16.43	1.41	1.21	4.29
16	8666	P6	32.50	21.45	2.51	1.00	1.00	1.01	1.00	1.00	0.51	1.21	4.29
17	8668	P1	21.49	16.97	1.01	15.00	14.49	0.81	11.49	1.00	0.21	1.36	6.11
18	8668	P3	17.97	14.97	1.01	19.97	12.96	1.01	13.49	1.00	-0.29	1.36	6.11
19	8668	P4	24.45	1.00	0.51	15.97	1.00	0.51	1.00	1.00	-0.09	1.36	6.11
20	8668	P6	20.45	17.44	1.51	12.96	15.49	1.51	1.00	1.00	0.51	1.36	6.11
21	8670	P1	27.39	22.36	2.41	29.93	19.00	1.81	14.49	1.00	2.01	1.00	7.35
22	8670	P3	41.95	31.98	3.01	36.51	26.98	1.51	24.37	15.97	0.61	1.00	7.35
23	8670	P4	34.41	27.93	3.61	30.98	24.68	1.81	29.00	16.97	1.81	1.00	7.35
24	8670	P6	34.00	27.46	2.01	41.47	23.49	2.21	23.00	14.00	1.61	1.00	7.35
25	8672	P1				17.00	15.97	0.91	13.49	10.49	0.61	1.18	4.55
26	8672	P3				45.17	15.49	1.21	21.49	1.00	0.21	1.18	4.55
27	8672	P4	40.42	28.98	3.31	40.95	18.89	2.21	26.98	14.49	0.71	1.18	4.55
28	8672	P6	31.46	27.84	2.81	27.39	17.97	2.31	14.97	12.49	0.81	1.18	4.55
29	8674	P1	31.22	43.50	4.81	32.98	37.88	4.01	1.00	1.00	-0.49	1.09	5.16
30	8674	P3	50.75	43.15	4.91	43.47	41.47	2.51	32.94	30.46	5.31	1.09	5.16
31	8674	P4	55.44	38.24	5.31	45.48	35.99	4.21	35.47	34.41	1.81	1.09	5.16
32	8674	P6	32.47	30.40	2.31	38.88	25.98	3.81	22.45	19.97	2.11	1.09	5.16
33	8676	P1	22.98	14.97	1.51	25.50	1.00	0.31	1.00	1.00	0.41	1.17	5.55
34	8676	P3	45.50	27.93	2.81	33.87	21.91	1.61	30.00	15.97	1.01	1.17	5.55
35	8676	P4	48.48	21.00	2.81	43.47	1.00	1.11	36.47	1.00	1.01	1.17	5.55
36	8676	P6	59.45	22.00	6.01	40.95	19.00	2.61	37.00	20.98	0.21	1.17	5.55
37	8680	P1	16.49	18.89	0.61	12.49	14.42	0.71	1.00	1.00	0.31	1.20	3.86
38	8680	P3	31.98	18.44	1.51	38.00	1.00	2.81	17.89	1.00	0.81	1.20	3.86
39	8680	P4	42.95	18.49	2.01	35.07	17.44	0.61	13.96	1.00	0.91	1.20	3.86
40	8680	P6	1.00	1.00	0.81	20.20	1.00	0.61	1.00	1.00	0.01	1.20	3.86
41	8684	P1	50.37	1.00	5.21	48.48	1.00	0.51	41.29	1.00	1.61	1.12	3.92
42	8684	P3	62.93	20.98	0.51	43.90	19.34	1.71	39.80	1.00	1.61	1.12	3.92
43	8684	P4	51.48	1.00	1.71	48.84	1.00	0.21	44.99	1.00	1.21	1.12	3.92
44	8684	P6	48.58	1.00	0.81	41.81	1.00	1.61	24.98	1.00	0.71	1.12	3.92
45	8710	P1	17.49	1.00	1.41	18.00	1.00	0.71	1.00	1.00	0.71	1.02	4.46
46	8710	P3	48.19	30.50	3.51	35.50	22.49	3.01	30.98	20.20	2.01	1.02	4.46
47	8710	P4	19.44	33.47	3.51	45.28	33.99	3.91	23.49	25.26	1.61	1.02	4.46
48	8710	P6	25.98	25.98	2.01	15.97	1.00	0.61	1.00	1.00	0.61	1.02	4.46
49	85321	P1	48.50	21.98	3.01	27.93	16.49	0.81	21.98	18.49	2.31	1.26	3.57
50	85321	P3	46.96	36.99	4.31	50.38	22.49	2.71	26.50	12.00	0.81	1.26	3.57

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30. min dil1	ERYTHEMA 4 h DIL 1	INC.THICK 4h DIL 1	OEDEMA 30 min DIL 2	ERYTHEA 4 h DIL 2	INC.THICK 4h DIL 2	OEDEMA 30 min DIL 3	ERYTHEMA 4 h DIL 3	INC.THICK 4H DIL 3	TRANS.SUSC EPT TEST 1	ELISA TEST 1
51	85321	P4	40.99	28.50	3.21	37.95	23.98	3.01	43.99	21.00	1.41	1.26	3.57
52	85321	P6	32.47	28.93	3.41	32.47	20.98	1.01	24.00	21.91	2.11	1.26	3.57
53	85379	P1	29.46	22.91	1.81	23.49	18.44	2.01	15.49	16.97	1.11	1.47	3.03
54	85379	P3	31.50	27.84	2.41	28.98	22.98	1.81	12.96	1.00	1.01	1.47	3.03
55	85379	P4	36.50	30.40	3.71	30.98	24.92	3.71	15.30	19.49	2.01	1.47	3.03
56	85379	P6	31.46	26.98	2.61	18.97	1.00	1.11	1.00	1.00	1.01	1.47	3.03
57	85381	P1	24.49	25.98	2.71	23.45	21.49	3.11	25.38	23.45	1.21	0.99	2.51
58	85381	P3	32.94	18.33	2.91	27.39	1.00	2.41	1.00	1.00	2.91	0.99	2.51
59	85381	P4	34.94	27.98	4.31	37.99	22.45	2.91	25.50	19.90	1.01	0.99	2.51
60	85381	P6	22.00	22.91	1.61	16.49	17.89	1.31	1.00	1.00	0.51	0.99	2.51
61	85395	P1	35.50	27.50	4.51	23.49	18.49	1.21	1.00	17.97	0.51	0.95	3.29
62	85395	P3	35.50	33.00	3.21	26.50	24.98	2.81	19.44	1.00	1.01	0.95	3.29
63	85395	P4	41.99	29.50	3.81	28.50	21.91	1.71	21.45	14.42	1.91	0.95	3.29
64	85395	P6	36.41	22.36	4.51	26.50	20.00	3.01	22.49	17.00	6.01	0.95	3.29
65	85397	P1	21.91	25.98	3.91	11.49	22.23	0.41	1.00	1.00	-0.49	0.64	5.65
66	85397	P3	24.92	33.99	3.51	21.49	23.98	3.01	1.00	1.00	1.01	0.64	5.65
67	85397	P4	35.47	33.99	5.31	38.99	32.94	5.01	37.50	31.98	1.91	0.64	5.65
68	85397	P6	35.47	24.45	3.31	23.00	19.97	3.81	1.00	1.00	0.91	0.64	5.65
69	86303	P1	36.95	29.39	3.71	21.98	20.49	2.01	1.00	16.00	1.21	0.90	2.36
70	86303	P3	36.00	28.93	4.31	18.97	1.00	1.31	1.00	1.00	1.01	0.90	2.36
71	86303	P4	32.94	24.98	4.01	19.00	19.49	1.51	19.44	14.97	0.61	0.90	2.36
72	86303	P6	16.43	21.00	1.41	15.00	13.49	0.51	1.00	1.00	0.21	0.90	2.36
73	86323	P1	29.93	26.98	7.01	30.46	17.49	-2.69	32.50	16.49	2.01	0.71	3.22
74	86323	P3	46.48	33.32	7.91	31.94	21.45	3.01	24.68	16.97	1.51	0.71	3.22
75	86323	P4	52.85	33.47	7.51	39.99	35.87	3.51	38.99	95.12	2.61	0.71	3.22
76	86323	P6	35.99	24.45	2.41	37.95	25.50	1.51	31.98	23.37	2.51	0.71	3.22
77	86333	P1	38.50	24.00	4.01	29.50	19.44	1.51	16.00	1.00	2.21	1.17	3.05
78	86333	P3	36.95	32.50	5.41	32.98	19.97	4.71	38.00	22.91	5.81	1.17	3.05
79	86333	P4	44.50	34.21	6.31	30.98	26.46	4.51	35.50	27.55	3.31	1.17	3.05
80	86333	P6	25.00	21.98	3.01	24.00	18.44	1.81	14.00	1.00	-0.39	1.17	3.05
81	86367	P1	54.99	20.00	4.11	34.47	14.97	2.31	21.45	1.00	1.71	1.22	3.34
82	86367	P3	47.92	19.97	2.91	41.99	13.96	1.91	29.93	12.85	1.31	1.22	3.34
83	86367	P4	43.27	22.98	3.81	39.80	30.98	3.01	30.94	17.89	2.31	1.22	3.34
84	86367	P6	36.33	18.44	3.51	23.45	1.00	1.71	11.96	1.00	0.31	1.22	3.34
85	86369	P1	27.98	1.00	2.01	17.49	1.00	1.61	14.49	1.00	2.11	1.27	3.55
86	86369	P3	40.50	29.50	2.61	32.00	20.45	1.31	22.00	1.00	0.81	1.27	3.55
87	86369	P4	38.95	24.68	2.81	33.32	24.49	2.51	15.00	1.00	1.01	1.27	3.55
88	86369	P6	15.97	23.98	1.41	14.49	19.90	0.81	10.00	1.00	0.51	1.27	3.55
89	86375	P1	35.85	22.36	1.81	27.46	18.00	2.31	1.00	1.00	1.91	1.09	3.39
90	86375	P3	45.43	23.98	2.01	31.46	20.49	1.51	27.46	16.97	0.91	1.09	3.39
91	86375	P4	47.43	18.44	1.01	47.96	26.98	2.31	27.93	1.00	-0.19	1.09	3.39
92	86375	P6	46.90	33.94	2.01	26.38	20.45	2.11	1.00	1.00	-0.19	1.09	3.39
93	86385	P1	28.98	22.98	2.51	24.49	19.97	1.11	14.49	9.95	-0.69	1.26	2.54
94	86385	P3	33.50	33.99	2.41	23.92	24.45	1.01	15.00	1.00	0.81	1.26	2.54
95	86385	P4	27.53	27.98	3.61	30.00	23.49	2.21	21.00	18.00	1.01	1.26	2.54
96	86385	P6	24.92	23.49	3.01	19.49	16.00	1.11	16.49	16.97	0.11	1.26	2.54
97	86389	P1	17.49	1.00	0.81	11.49	1.00	-0.19	1.00	1.00	0.71	0.45	2.59
98	86389	P3	25.00	19.34	2.01	16.97	13.49	2.71	14.00	1.00	0.81	0.45	2.59
99	86389	P4	22.00	26.50	1.81	22.49	19.49	2.91	20.49	1.00	1.31	0.45	2.59
100	86389	P6	21.98	23.98	3.71	21.49	14.42	0.61	15.00	1.00	0.91	0.45	2.59
101	86511	P1				18.00	15.49	1.91	1.00	1.00	-1.49	0.92	3.42

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30. min dil1	ERYTHEMA 4 h DIL 1	INC.THICK 4h DIL 1	OEDEMA 30 min DIL 2	ERYTHEA 4 h DIL 2	INC.THICK 4h DIL 2	OEDEMA 30 min DIL 3	ERYTHEMA 4 h DIL 3	INC.THICK 4H DIL 3	TRANS.SUSC EPT TEST 1	ELISA TEST 1
102	86511	P3	38.99	27.98	4.51	23.49	15.49	2.01	21.49	18.97	1.31	0.92	3.42
103	86511	P4	44.90	39.47	5.81	35.31	26.53	2.71	27.98	17.75	2.01	0.92	3.42
104	86511	P6	30.50	25.46	4.51	18.44	16.03	2.11	15.00	1.00	1.01	0.92	3.42
105	8660	GSG	30.50	26.38	3.81							0.63	5.68
106	8662	GSG	36.47	32.98	7.51							1.09	9.39
107	8664	GSG	22.98	25.51	4.21							1.37	3.37
108	8666	GSG	31.46	22.49	3.21							1.21	4.29
109	8668	GSG	21.49	27.46	3.71							1.36	6.11
110	8670	GSG	27.46	25.92	3.21							1.00	7.35
111	8672	GSG	27.84	26.50	3.31							1.18	4.55
112	8674	GSG	47.43	13.11	6.71							1.09	5.16
113	8676	GSG	40.80	25.50	3.51							1.17	5.55
114	8680	GSG	28.98	21.49	1.41							1.20	3.86
115	8684	GSG	33.47	19.44	2.81							1.12	3.92
116	8702	GSG										1.35	5.10
117	8708	GSG										1.20	5.80
118	8710	GSG	20.49	33.87	3.01							1.02	4.46
119	8712	GSG										1.21	5.45
120	8716	GSG										1.46	5.76
121	8718	GSG										1.40	4.79
122	8722	GSG										1.24	3.99
123	85321	GSG	45.00	38.79	3.31							1.26	3.57
124	85379	GSG	32.94	34.94	3.71							1.47	3.03
125	85381	GSG	42.50	29.98	6.81							0.99	2.51
126	85395	GSG	39.50	26.50	3.81							0.95	3.29
127	85397	GSG	44.99	38.42	7.51							0.64	5.65
128	85405	GSG										0.55	5.13
129	86303	GSG	33.00	30.98	5.61							0.90	2.36
130	86323	GSG	43.43	36.33	8.51							0.71	3.22
131	86333	GSG	37.47	31.86	3.51							1.17	3.05
132	86367	GSG	35.50	31.46	4.81							1.22	3.34
133	86369	GSG	33.94	20.90	2.11							1.27	3.55
134	86375	GSG	38.11	36.47	3.71							1.09	3.39
135	86385	GSG	28.93	24.49	3.71							1.26	2.54
136	86389	GSG	27.98	27.98	4.21							0.45	2.59
137	86511	GSG	31.02	22.98	5.01							0.92	3.42

DATASET : DOSHET-2.Chapter nine.TEST 2

CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 M DIL-1	ERYT 4 H DIL-1	INC THICK 4HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H IL-2	D INC THICK 4H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DI L 3	INC THICK 4H DIL-3	MEAN TICK COUNT.LOG	ELISA TEST 2
1	8660	P3	20.49	22.00	2.41	1.00	1.00	1.01	1.00	1.00	-1.39	2.19	5.03
2	8660	P6	23.98	18.97	1.01	17.44	14.97	0.31	1.00	1.00	0.51	2.19	5.03
3	8660	P5	22.98	29.73	0.61	25.50	1.00	1.51	21.49	20.49	0.71	2.19	5.03
4	8660	GSG	22.49	22.49	4.81	30.98	21.98	2.91	23.49	18.49	1.81	2.19	5.03
5	8662	P3	20.49	24.68	4.01	14.97	1.00	-1.39	8.00	1.00	-0.49	1.89	7.16
6	8662	P6	27.50	27.98	5.31	17.97	19.44	1.61	12.49	12.41	-0.89	1.89	7.16
7	8662	P5	32.47	22.45	2.91	26.50	13.42	2.51	22.98	11.83	2.01	1.89	7.16
8	8662	GSG	26.98	22.49	4.01	22.49	20.98	3.71	22.91	12.96	1.41	1.89	7.16

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 M DIL-1	ERYT 4 H DIL-1	INC THICK 4HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H D IL-2	INC THICK 4H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DI L 3	INC THICK 4H DIL-3	MEAN TICK COUNT.LOG	ELISA TEST 2
9	8664	P3	12.00	1.00	1.11	24.49	1.00	1.21	15.49	1.00	0.61	2.35	4.66
10	8664	P6	25.00	20.45	0.71	13.96	1.00	-0.39	1.00	1.00	0.11	2.35	4.66
11	8664	P5	30.94	22.49	3.41	17.97	1.00	1.31	17.00	1.00	-0.09	2.35	4.66
12	8664	GSG	1.00	25.50	2.71	31.94	14.42	1.41	14.97	1.00	1.51	2.35	4.66
13	8666	P3	23.98	20.45	2.01	18.44	20.00	1.61	15.49	1.00	0.71	1.88	5.67
14	8666	P6	29.29	23.49	3.01	14.97	1.00	0.61	10.49	1.00	0.41	1.88	5.67
15	8666	P5	32.65	26.46	1.71	16.00	1.00	1.11	15.49	1.00	1.61	1.88	5.67
16	8666	GSG	32.31	30.98	3.11	27.50	24.98	1.51	24.00	19.49	1.61	1.88	5.67
17	8668	P3	23.81	15.43	0.41	20.45	16.97	1.81	1.00	1.00	0.71	2.59	6.13
18	8668	P6	24.37	16.73	-0.49	16.49	1.00	-0.79	9.49	1.00	1.11	2.59	6.13
19	8668	P5	24.00	18.97	1.41	23.45	13.49	2.01	9.49	1.00	0.71	2.59	6.13
20	8668	GSG	28.50	25.98	2.61	22.98	20.45	3.11	18.97	15.97	0.61	2.59	6.13
21	8670	P3	24.98	26.46	2.81	21.00	15.97	1.11	19.49	1.00	0.61	1.87	6.00
22	8670	P6	23.98	23.98	1.01	19.97	15.97	2.41	15.97	1.00	0.81	1.87	6.00
23	8670	P5	35.50	32.31	5.01	26.98	21.45	2.71	29.00	18.89	4.91	1.87	6.00
24	8670	GSG	30.98	30.94	6.81	28.46	20.98	5.21	24.98	18.97	3.01	1.87	6.00
25	8672	P3	1.00	12.41	1.61	16.00	11.49	0.91	1.00	1.00	1.11	1.89	5.29
26	8672	P6	22.49	15.97	3.31	1.00	12.96	1.01	1.00	9.95	-1.09	1.89	5.29
27	8672	P5	26.46	19.44	1.21	21.35	12.96	1.31	19.00	12.41	0.51	1.89	5.29
28	8672	GSG	15.49	23.45	3.61	20.49	19.49	3.31	22.91	14.49	1.61	1.89	5.29
29	8674	P3	20.98	18.49	2.21	14.97	12.49	0.61	1.00	1.00	1.01	1.95	5.21
30	8674	P6	27.46	16.61	-1.09	6.32	15.43	-0.59	15.87	12.41	-0.59	1.95	5.21
31	8674	P5	32.98	22.91	2.01	22.98	16.97	3.81	14.49	17.49	1.81	1.95	5.21
32	8674	GSG	39.50	22.49	6.41	36.95	24.82	5.51	19.97	15.49	2.71	1.95	5.21
33	8676	P3	17.97	15.00	2.31	16.97	13.49	2.01	12.49	11.49	1.11	2.18	6.61
34	8676	P6	20.45	16.12	1.51	18.97	14.42	0.41	12.00	12.00	0.51	2.18	6.61
35	8676	P5	24.68	20.98	4.31	20.49	10.95	1.31	19.44	12.25	1.51	2.18	6.61
36	8676	GSG	26.50	22.91	5.31	21.91	17.89	4.71	22.98	10.20	3.31	2.18	6.61
37	8680	P3	16.00	14.49	1.01	10.49	11.96	-0.09	9.00	1.00	-0.39	1.86	4.79
38	8680	P6	19.97	13.49	1.11	14.49	11.00	0.51	7.48	1.00	0.41	1.86	4.79
39	8680	P5	23.98	21.35	2.21	16.49	12.96	1.31	15.49	13.49	0.71	1.86	4.79
40	8680	GSG	33.50	22.36	4.11	23.49	17.32	1.31	13.49	10.95	0.21	1.86	4.79
41	8684	P3	1.00	13.00	1.21	16.49	12.49	2.01	1.00	1.00	1.21	2.25	5.48
42	8684	P6	20.35	15.43	1.81	23.00	14.49	1.41	15.49	1.00	0.71	2.25	5.48
43	8684	P5	39.12	20.49	3.21	26.27	13.49	1.81	33.50	15.97	1.41	2.25	5.48
44	8684	GSG	35.94	19.49	5.71	36.00	16.49	2.71	28.46	15.43	2.11	2.25	5.48
45	8702	P3	18.49	1.00	0.71	12.49	1.00	0.61	1.00	1.00	-0.59	2.34	5.21
46	8702	P6	18.97	10.39	0.11	9.95	1.00	-0.79	1.00	1.00	-0.39	2.34	5.21
47	8702	P5	19.90	11.40	1.01	12.00	1.00	-0.59	7.00	1.00	0.51	2.34	5.21
48	8702	GSG	23.92	13.49	1.61	28.93	10.00	-0.99	17.00	1.00	-0.39	2.34	5.21
49	8708	P3	21.91	27.50	2.01	19.49	13.42	1.81	1.00	1.00	0.21	2.34	6.14
50	8708	P6	21.98	18.89	4.01	1.00	1.00	-0.19	1.00	1.00	-0.49	2.34	6.14
51	8708	P5	25.98	13.00	0.71	19.00	1.00	0.91	26.50	1.00	-0.49	2.34	6.14
52	8708	GSG	24.98	20.98	3.31	21.49	12.96	2.11	17.00	1.00	1.01	2.34	6.14
53	8710	P3	34.50	25.38	1.31	24.49	16.97	2.01	1.00	1.00	0.51	1.75	5.88
54	8710	P6	22.45	14.49	1.01	17.49	1.00	0.11	12.00	1.00	-0.19	1.75	5.88
55	8710	P5	31.98	18.97	2.01	24.45	10.39	1.41	13.96	1.00	1.01	1.75	5.88
56	8710	GSG	25.50	23.98	3.01	23.49	17.89	4.21	22.98	13.27	2.21	1.75	5.88
57	8712	P3	21.00	9.49	1.61	18.49	1.00	1.21	1.00	1.00	0.11	2.22	6.10
58	8712	P6	21.00	1.00	1.01	1.00	1.00	0.51	1.00	1.00	0.61	2.22	6.10
59	8712	P5	24.92	15.00	2.01	1.00	1.00	0.91	1.00	1.00	0.51	2.22	6.10

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 H DIL-1	ERYT 4 H DIL-1	INC THICK 4HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H IL-2	D INC THICK 4H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DI L 3	INC THICK 4H DIL-3	MEAN TICK COUNT.LOG	ELISA TEST 2
60	8712	GSG	1.00	14.00	1.51	1.00	1.00	0.41	16.49	1.00	0.31	2.22	6.10
61	8716	P3	14.00	11.49	-0.79	22.45	12.96	0.81	1.00	1.00	-0.69	2.39	6.97
62	8716	P6	22.00	11.40	0.51	19.00	1.00	0.01	1.00	1.00	0.01	2.39	6.97
63	8716	P5	20.49	15.87	1.11	18.97	1.00	0.01	13.49	1.00	0.61	2.39	6.97
64	8716	GSG	18.49	16.97	2.11	17.89	18.97	2.51	13.42	11.96	1.21	2.39	6.97
65	8718	P3				26.93	21.98	4.11	22.00	18.17	1.81	2.31	6.02
66	8718	P6	17.97	15.43	1.11	12.41	11.83	-0.09	8.00	8.94	0.71	2.31	6.02
67	8718	P5	22.00	16.49	2.61	19.00	11.96	1.81	13.49	10.00	0.61	2.31	6.02
68	8718	GSG	27.93	28.50	5.11	24.49	16.43	2.71	16.97	11.49	3.11	2.31	6.02
69	8722	P3				14.49	9.80	2.21	10.00	9.00	2.21	2.16	
70	8722	P6	23.98	15.43	2.61	20.49	13.86	1.01	11.96	1.00	-0.09	2.16	
71	8722	P5	25.50	23.49	4.71	17.49	10.39	2.21	7.48	8.00	0.71	2.16	
72	8722	GSG	22.98	26.00	6.21	18.44	14.87	2.51	16.00	10.95	1.41	2.16	
73	85321	P3	33.41	17.44	-2.39	23.49	14.70	2.81	15.97	9.49	0.91	2.27	3.62
74	85321	P6	45.48	19.18	1.21	24.98	13.96	1.51	10.95	9.95	-0.09	2.27	3.62
75	85321	P5	41.95	26.46	5.51	31.98	15.49	4.91	28.98	20.49	1.71	2.27	3.62
76	85321	GSG	35.99	20.98	2.21	42.95	18.49	2.21	27.46	12.96	3.01	2.27	3.62
77	85379	P3	37.52	1.00	1.61	20.98	20.45	0.51	1.00	1.00	0.61	2.40	5.05
78	85379	P6	22.45	24.98	3.71	18.49	19.00	1.41	8.49	1.00	1.91	2.40	5.05
79	85379	P5	33.99	24.98	5.01	26.50	20.98	4.01	19.00	14.97	1.81	2.40	5.05
80	85379	GSG	37.47	31.40	5.61	33.00	25.69	2.51	39.50	29.39	4.41	2.40	5.05
81	85381	P3				28.46	25.46	3.71	25.98	16.97	4.41	1.88	3.65
82	85381	P6	20.98	20.00	3.51	13.96	14.97	-1.49	1.00	1.00	0.71	1.88	3.65
83	85381	P5	38.99	32.50	4.41	28.50	18.97	1.61	14.07	14.28	0.61	1.88	3.65
84	85381	GSG	32.40	26.98	5.41	26.50	23.49	4.61	22.49	15.87	3.41	1.88	3.65
85	85395	P3				17.44	16.43	4.31	14.42	13.86	1.51	2.14	4.67
86	85395	P6	24.49	21.45	3.01	14.97	16.43	0.41	10.49	9.00	0.01	2.14	4.67
87	85395	P5	29.46	29.98	5.51	25.00	16.97	1.91	13.00	11.00	3.31	2.14	4.67
88	85395	GSG	32.47	30.94	5.51	25.50	20.49	3.81	23.92	13.96	1.71	2.14	4.67
89	85397	P3	38.88	46.83	9.01	43.90	36.22	5.51	34.00	32.47	4.31	2.08	4.36
90	85397	P6	42.99	33.47	4.81	27.13	25.98	2.81	14.49	17.97	1.41	2.08	4.36
91	85397	P5	45.50	22.45	5.71	31.94	26.38	3.41	20.98	18.00	2.31	2.08	4.36
92	85397	GSG	39.34	30.40	5.51	44.00	28.98	5.51	28.00	24.25	1.51	2.08	4.36
93	85405	P3	23.45	21.49	2.21	22.00	15.97	3.41	14.97	12.96	0.41	1.10	5.46
94	85405	P6	36.99	29.50	2.71	21.49	16.00	0.51	14.42	15.49	0.01	1.10	5.46
95	85405	P5	48.84	23.37	3.81	33.50	19.97	2.11	28.39	16.97	2.51	1.10	5.46
96	85405	GSG	32.94	18.44	2.61	30.98	17.49	3.51	25.98	11.96	1.31	1.10	5.46
97	86303	P3	17.97	16.31	2.01	1.00	13.86	3.31	1.00	9.95	0.41	1.85	3.90
98	86303	P6	19.44	13.86	1.41	11.00	12.00	2.51	1.00	1.00	1.71	1.85	3.90
99	86303	P5	27.46	12.49	2.11	16.97	11.96	1.21	8.00	9.00	0.81	1.85	3.90
100	86303	GSG	23.37	17.00	3.51	27.46	22.45	4.11	17.97	14.49	2.51	1.85	3.90
101	86323	P3	38.00	19.97	-5.29	26.46	13.42	3.41	12.49	12.85	1.81	1.79	4.44
102	86323	P6	30.46	20.45	2.71	26.50	1.00	-1.19	12.49	1.00	0.01	1.79	4.44
103	86323	P5	29.50	25.98	8.11	21.98	16.49	5.01	25.38	19.18	2.01	1.79	4.44
104	86323	GSG	42.50	29.85	6.91	35.41	19.44	2.51	32.50	12.41	3.51	1.79	4.44
105	86333	P3	20.98	15.87	2.01	19.97	15.49	3.11	17.44	12.00	-0.89	2.29	4.28
106	86333	P6	26.38	18.57	2.01	17.49	12.00	1.01	10.95	11.83	2.71	2.29	4.28
107	86333	P5	27.46	14.97	3.01	23.45	12.00	4.01	15.49	1.00	0.71	2.29	4.28
108	86333	GSG	28.98	19.44	2.41	27.93	17.89	1.91	25.46	10.49	1.81	2.29	4.28
109	86367	P3	48.44	15.49	4.81	33.41	11.00	3.61	30.94	12.96	2.31	2.40	4.67
110	86367	P6	34.94	18.33	2.31	11.49	1.00	0.41	24.45	11.96	2.81	2.40	4.67

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 M DIL-1	ERYT 4 H DIL-1	INC THICK 4HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H D IL-2	INC THICK 4H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DI L 3	INC THICK 4H DIL-3	MEAN TICK COUNT.LOG	ELISA TEST 2
111	86367	P5	41.95	24.98	3.81	26.46	21.98	1.61	19.44	14.28	1.61	2.40	4.67
112	86367	GSG	28.98	22.49	2.01	27.93	14.49	2.31	28.46	15.43	2.21	2.40	4.67
113	86369	P3	14.49	19.97	1.21	12.00	1.00	1.01	1.00	1.00	0.51	2.29	4.71
114	86369	P6	13.49	11.96	1.31	1.00	1.00	0.31	1.00	1.00	0.41	2.29	4.71
115	86369	P5	31.94	19.44	2.31	18.44	17.89	1.61	1.00	1.00	0.91	2.29	4.71
116	86369	GSG	20.00	23.45	3.61	23.49	15.97	1.61	17.44	20.00	2.11	2.29	4.71
117	86375	P3	22.98	15.97	2.81	25.50	1.00	2.01	1.00	1.00	0.21	1.91	4.85
118	86375	P6	27.50	24.45	2.91	19.97	16.73	0.31	1.00	1.00	0.91	1.91	4.85
119	86375	P5	40.00	34.50	2.61	25.98	19.00	1.71	27.50	18.89	1.21	1.91	4.85
120	86375	GSG	21.45	16.97	3.51	28.46	16.88	2.41	31.40	22.45	2.01	1.91	4.85
121	86385	P3	33.32	23.49	3.81				17.97	12.41	1.61	1.84	4.00
122	86385	P6	24.92	19.49	2.91	20.98	15.97	1.41	13.96	15.30	1.31	1.84	4.00
123	86385	P5	34.50	24.98	3.51	22.45	14.00	2.21	16.97	11.22	1.31	1.84	4.00
124	86385	GSG	27.55	24.49	5.71	27.50	21.91	5.01	21.49	16.97	2.11	1.84	4.00
125	86389	P3	33.50	22.49	2.21	28.98	20.49	3.01	24.45	20.49	5.01	1.08	3.44
126	86389	P6	23.00	16.88	2.91	18.97	17.29	1.01	12.96	12.49	-1.09	1.08	3.44
127	86389	P5	31.46	24.49	3.31	25.50	17.44	1.41	18.49	13.49	2.91	1.08	3.44
128	86389	GSG	32.40	27.98	6.51	35.99	25.50	3.71	27.46	13.96	0.81	1.08	3.44
129	86511	P3	24.00	15.97	2.81	18.49	14.00	3.21	1.00	1.00	1.71	1.82	4.59
130	86511	P6	30.98	16.43	1.31	21.98	1.00	1.21	14.97	1.00	-0.99	1.82	4.59
131	86511	P5	31.40	23.45	2.61	27.46	16.97	2.31	21.98	12.96	1.41	1.82	4.59
132	86511	GSG	32.40	23.07	2.61	28.50	25.50	3.11	27.93	15.49	2.31	1.82	4.59

DATASET : DOSHET-3.Chapter nine. test 3

CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 M DIL-1	ERYT 4 H DIL-1	INC THICK 4HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H DIL-2	INC THICK 4H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DIL 3	INC THICK 4H DIL-3	TRANS.SUSC .TEST 3	ELISA TEST 3
1	8660	P3	38.42	26.98	4.51	30.50	20.98	2.11	19.44	14.49	0.61	1.46	4.91
2	8660	P4	44.27	26.93	2.91	30.94	22.49	2.71	25.50	16.97	0.01	1.46	4.91
3	8660	P5	39.00	27.28	1.61	31.94	24.98	1.41	14.97	16.49	0.21	1.46	4.91
4	8660	GSG	37.23	20.98	2.81	27.50	22.98	0.81	11.49	15.49	0.71	1.46	4.91
5	8662	P3	43.71	29.46	4.01	30.85	26.83	2.41	18.49	19.90	1.31	1.29	6.07
6	8662	P4	43.47	27.39	4.21	29.98	23.45	3.31	30.46	28.84	2.61	1.29	6.07
7	8662	P5	32.47	26.83	4.01	25.98	20.98	2.21	26.46	19.97	1.01	1.29	6.07
8	8662	GSG	48.44	27.98	4.71	36.50	19.44	2.81	33.47	17.89	1.21	1.29	6.07
9	8664	P3	46.04	22.36	3.21	38.96	23.98	2.01	29.93	16.43	1.41	1.55	3.34
10	8664	P4	48.37	32.50	4.11	38.50	23.45	2.71	29.98	24.45	2.91	1.55	3.34
11	8664	P5	42.90	22.91	3.61	32.50	17.89	3.01	18.97	14.00	1.61	1.55	3.34
12	8664	GSG	45.50	34.50	8.21	40.47	35.50	5.81	23.00	13.96	1.61	1.55	3.34
13	8666	P3	42.36	25.38	4.21	41.50	19.90	2.31	24.49	14.49	1.01	1.21	4.65
14	8666	P4	53.44	31.98	2.81	36.47	18.44	1.91	25.00	10.95	0.71	1.21	4.65
15	8666	P5	39.76	13.96	1.81	27.50	11.49	0.91	23.92	13.86	0.81	1.21	4.65
16	8666	GSG	29.39	20.49	3.31	38.47	23.49	2.61	28.00	12.96	1.21	1.21	4.65
17	8668	P3	30.85	36.00	1.01	29.50	30.46	2.41	27.46	18.49	1.51	1.98	5.34
18	8668	P4	35.07	33.50	4.81	28.84	31.50	2.81	28.46	23.92	1.81	1.98	5.34
19	8668	P5	30.98	23.49	4.11	26.98	22.91	1.91	21.98	14.49	0.91	1.98	5.34
20	8668	GSG	25.98	22.49	2.41	18.97	18.97	3.01	14.97	13.49	0.51	1.98	5.34
21	8670	P3	31.86	27.50	5.01	32.47	24.49	2.91	9.38	23.00	3.71	1.21	5.96
22	8670	P4	35.94	38.42	6.61	28.98	25.98	4.51	21.45	22.45	1.51	1.21	5.96
23	8670	P5	38.50	31.50	5.71	29.46	23.98	4.41	12.41	16.00	0.21	1.21	5.96

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 H DIL-1	ERYT 4 H DIL-1	INC THICK 4HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H DIL-2	INC THICK 4H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DIL 3	INC THICK 4H DIL-3	TRANS.SUSC .TEST 3	ELISA TEST 3
24	8670	GSG	32.98	28.28	5.51	28.98	23.45	3.11	19.49	16.49	3.21	1.21	5.96
25	8672	P3	31.98	37.99	3.21	28.50	26.98	3.01	23.45	16.97	1.81	1.43	3.49
26	8672	P4	40.35	38.24	4.81	27.46	29.50	4.41	21.45	24.45	1.51	1.43	3.49
27	8672	P5	27.93	28.98	3.71	23.98	16.97	2.01	23.92	19.97	1.41	1.43	3.49
28	8672	GSG	36.22	29.73	3.81	25.50	22.49	3.01	18.49	16.49	1.41	1.43	3.49
29	8674	P3	43.15	43.99	3.81	37.42	35.78	1.81	28.72	21.79	1.51	1.52	4.43
30	8674	P4	40.42	40.99	6.01	35.41	31.94	3.51	33.94	34.94	1.91	1.52	4.43
31	8674	P5	43.99	32.94	3.31	25.98	24.45	1.01	14.00	23.45	1.11	1.52	4.43
32	8674	GSG	43.00	28.84	5.51	39.00	22.23	3.51	25.00	14.49	1.21	1.52	4.43
33	8676	P3	41.99	20.98	2.11	34.41	15.87	0.91	31.46	14.00	1.21	1.70	5.96
34	8676	P4	44.90	26.98	4.21	29.46	15.97	1.61	27.50	15.97	2.41	1.70	5.96
35	8676	P5	35.94	26.50	3.31	29.93	17.44	2.11	21.45	13.49	2.01	1.70	5.96
36	8676	GSG	42.99	24.00	3.61	29.29	18.97	2.51	20.45	12.96	0.61	1.70	5.96
37	8680	P3	41.89	16.97	1.41	30.00	18.00	0.91	22.98	9.95	0.01	1.34	4.25
38	8680	P4	65.99	24.98	4.01	54.50	21.91	2.21	30.50	12.96	0.81	1.34	4.25
39	8680	P5	40.99	23.49	1.41	26.50	15.00	0.71	33.27	12.96	-0.39	1.34	4.25
40	8680	GSG	36.93	19.44	1.41	30.50	16.97	1.21	14.87	10.49	-0.19	1.34	4.25
41	8684	P3	53.44	28.50	5.21	42.99	22.00	3.71	34.99	16.00	1.61	1.44	3.69
42	8684	P4	49.44	21.35	3.51	39.89	20.49	3.91	33.41	18.00	3.51	1.44	3.69
43	8684	P5	30.50	13.96	2.21	33.00	12.49	1.81	31.40	11.49	1.81	1.44	3.69
44	8684	GSG	43.50	21.45	3.31	40.99	16.49	2.31	31.46	10.49	1.51	1.44	3.69
45	8702	P3	38.47	15.87	2.31	32.86	12.96	1.21	18.89	9.49	-0.99	1.59	3.97
46	8702	P4	41.95	18.49	1.71	31.50	15.49	2.01	20.49	13.49	0.71	1.59	3.97
47	8702	P5	40.00	21.45	2.31	26.46	15.00	1.61	21.91	13.00	1.51	1.59	3.97
48	8702	GSG	40.99	25.92	3.51	29.46	16.97	2.41	13.49	12.49	1.01	1.59	3.97
49	8708	P3	33.00	21.91	5.51	37.99	16.49	2.61	20.49	10.49	1.31	1.56	5.85
50	8708	P4	45.99	41.50	6.81	42.47	24.92	3.71	27.50	19.49	2.01	1.56	5.85
51	8708	P5	33.99	25.00	4.81	28.98	20.78	2.11	27.84	18.97	1.41	1.56	5.85
52	8708	GSG	39.00	30.98	4.01	27.46	18.97	3.11	22.49	14.49	2.81	1.56	5.85
53	8710	P3				45.72	1.00	0.21	32.12	10.49	1.11	1.48	5.06
54	8710	P4	47.50	18.44	2.71	32.86	13.42	1.51	27.28	11.96	1.21	1.48	5.06
55	8710	P5	31.50	13.96	2.21	21.79	11.96	1.61	20.35	12.96	1.71	1.48	5.06
56	8710	GSG	30.50	17.97	2.51	18.89	14.70	1.01	16.43	9.95	0.61	1.48	5.06
57	8712	P3	35.78	18.97	2.51	39.00	14.97	1.11	19.90	14.87	2.01	1.59	5.86
58	8712	P4	35.99	20.90	1.81	28.46	19.90	1.21	19.97	16.49	0.71	1.59	5.86
59	8712	P5	10.39	1.00	-0.29	21.00	8.77	1.01	10.39	9.49	0.51	1.59	5.86
60	8712	GSG	33.47	16.49	2.01	23.37	9.95	1.61	13.96	1.00	0.91	1.59	5.86
61	8716	P3				31.98	18.97	1.21	22.98	9.49	0.51	1.55	5.45
62	8716	P4	31.94	21.98	2.71				22.91	11.00	0.31	1.55	5.45
63	8716	P5	32.00	18.97	1.91				11.49	9.49	0.11	1.55	5.45
64	8716	GSG	22.98	14.28	1.51	22.49	13.42	2.01	14.97	12.00	0.61	1.55	5.45
65	8718	P3	36.99	40.50	2.61	32.86	35.99	2.91	32.47	28.84	2.51	1.64	5.56
66	8718	P4	53.85	51.21	4.71	35.94	37.79	4.21	34.47	27.28	3.01	1.64	5.56
67	8718	P5	30.98	27.00	2.91	23.45	19.34	2.71	14.49	14.97	2.31	1.64	5.56
68	8718	GSG	38.50	37.99	4.01	26.50	19.97	2.51	16.97	13.49	2.11	1.64	5.56
69	8722	P3	37.88	32.02	3.31	36.99	24.49	3.31	29.50	16.97	3.01	1.55	5.74
70	8722	P4	46.96	29.50	5.41	34.50	27.46	4.01	26.50	16.43	3.81	1.55	5.74
71	8722	P5	40.42	28.39	5.01	35.99	21.98	4.61	22.98	14.97	2.51	1.55	5.74
72	8722	GSG	38.42	30.46	5.61	32.94	26.50	4.41	19.97	16.43	3.71	1.55	5.74
73	85321	P3	40.00	18.97	4.51	36.47	15.49	2.31	26.93	13.42	1.71	1.87	2.34
74	85321	P4	42.95	22.98	3.01	36.47	15.87	1.01	24.49	9.49	0.71	1.87	2.34

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 M DIL-1	ERYT 4 H DIL-1	INC THICK 4HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H DIL-2	INC THICK 4H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DIL 3	INC THICK 4H DIL-3	TRANS.SUSC .TEST 3	ELISA TEST 3
75	85321	P5	29.58	20.49	3.51	27.50	16.49	2.31	17.49	11.49	0.01	1.87	2.34
76	85321	GSG	40.47	27.39	5.21	35.50	25.92	4.21	23.00	15.00	0.91	1.87	2.34
77	85379	P3	36.41	40.35	2.21	30.46	31.40	2.91	24.00	24.82	1.71	2.10	3.68
78	85379	P4	56.92	48.29	6.21	38.88	33.47	2.91	22.98	27.39	1.61	2.10	3.68
79	85379	P5	39.99	38.11	4.61	33.94	29.00	0.91	18.49	19.44	0.01	2.10	3.68
80	85379	GSG	40.80	41.47	6.31	42.71	39.47	7.41	35.33	23.92	1.01	2.10	3.68
81	85381	P3	39.95	46.00	6.21	37.88	34.50	4.91	41.42	27.93	2.21	1.57	3.05
82	85381	P4	50.96	45.96	5.01	51.96	37.95	6.11	38.99	29.46	3.21	1.57	3.05
83	85381	P5	41.47	45.50	3.61	23.92	22.45	2.31	12.96	14.97	0.41	1.57	3.05
84	85381	GSG	45.11	36.95	7.51	33.47	32.50	4.51	21.35	19.00	1.81	1.57	3.05
85	85395	P3	45.83	38.95	7.01	41.99	24.49	3.61	20.00	16.97	2.01	1.84	4.16
86	85395	P4	56.48	43.95	7.31	41.50	26.00	4.31	28.46	18.89	4.11	1.84	4.16
87	85395	P5	52.76	35.41	7.41	35.00	24.37	7.31	15.97	12.96	2.21	1.84	4.16
88	85395	GSG	35.94	33.63	6.01	34.32	25.98	3.21	21.49	13.96	2.91	1.84	4.16
89	85397	P3	52.92	47.29	11.21	51.50	21.98	4.31	46.48	18.97	2.61	1.59	3.78
90	85397	P4	63.72	56.86	11.61	51.21	31.30	4.01	47.48	28.72	4.01	1.59	3.78
91	85397	P5	50.91	25.92	4.51	34.94	16.49	1.91	25.46	16.97	1.81	1.59	3.78
92	85397	GSG	54.48	34.47	6.51	47.50	29.00	4.51	38.50	22.36	1.71	1.59	3.78
93	85405	P3	56.44	31.86	5.31	34.06	20.98	3.01	28.46	18.97	1.61	1.12	4.83
94	85405	P4	46.99	29.50	5.81	36.88	23.49	3.51	32.40	22.49	0.61	1.12	4.83
95	85405	P5	37.47	33.47	8.51	25.46	23.45	3.31	18.49	16.43	-0.69	1.12	4.83
96	85405	GSG	35.00	30.00	6.91	30.46	29.00	6.31	18.97	14.49	1.91	1.12	4.83
97	86303	P3	36.50	16.49	2.51	25.50	13.96	2.31	14.97	13.49	0.21	1.54	2.36
98	86303	P4	26.00	22.49	3.71	24.45	17.44	1.81	19.90	13.96	1.11	1.54	2.36
99	86303	P5	18.97	17.97	2.21	27.84	18.49	3.21	15.43	13.96	1.71	1.54	2.36
100	86303	GSG	34.50	21.49	5.21	21.49	14.49	1.11	23.00	11.96	1.71	1.54	2.36
101	86323	P3	47.50	36.47	4.01	40.42	33.94	3.51	40.89	30.98	4.01	1.66	4.03
102	86323	P4	44.99	40.99	5.01	34.94	39.50	4.31	33.41	32.98	2.11	1.66	4.03
103	86323	P5	41.42	33.99	5.31	39.95	28.98	4.31	23.00	22.49	3.31	1.66	4.03
104	86323	GSG	40.50	43.99	5.51	34.94	32.86	6.51	21.45	18.00	1.51	1.66	4.03
105	86333	P3	39.47	26.46	2.21	32.40	20.98	1.41	25.50	15.87	1.81	1.86	3.75
106	86333	P4	50.38	29.93	2.91	31.46	20.98	0.21	34.47	16.97	0.41	1.86	3.75
107	86333	P5	38.99	26.46	2.61	33.99	20.35	0.51	24.00	14.00	1.01	1.86	3.75
108	86333	GSG	41.89	23.00	1.51	27.50	16.97	1.01	17.00	12.41	0.01	1.86	3.75
109	86367	P3	34.41	33.47	4.01	33.50	24.45	3.41	26.38	22.45	3.21	1.67	4.68
110	86367	P4	53.48	35.94	6.41	40.89	25.69	2.81	39.99	22.36	1.81	1.67	4.68
111	86367	P5	45.46	29.39	2.71	39.50	22.36	1.21	28.98	15.49	1.31	1.67	4.68
112	86367	GSG	43.47	32.47	2.61	32.50	29.50	3.01	18.49	11.49	2.21	1.67	4.68
113	86369	P3	43.95	28.93	2.61	33.50	18.49	2.01	14.49	11.49	1.21	1.82	4.53
114	86369	P4	50.44	21.63	2.81	30.98	15.87	2.41	20.98	11.49	1.61	1.82	4.53
115	86369	P5	37.47	17.97	3.01	23.98	12.49	0.81	12.96	10.49	0.01	1.82	4.53
116	86369	GSG	37.99	20.49	3.91	25.98	15.97	2.41	18.97	11.49	1.21	1.82	4.53
117	86375	P3	45.90	31.50	4.61	35.50	28.46	3.31	23.49	21.35	1.31	1.51	3.72
118	86375	P4	34.50	38.78	5.01	34.94	28.50	4.61	32.00	22.49	3.01	1.51	3.72
119	86375	P5	33.87	24.82	2.71	32.45	24.49	3.61	22.91	18.76	2.71	1.51	3.72
120	86375	GSG	40.95	30.98	6.31	38.47	28.50	6.51	23.49	17.97	0.71	1.51	3.72
121	86385	P3	39.24	34.90	2.01	31.50	19.49	1.01	22.98	16.88	0.51	1.69	3.51
122	86385	P4	38.99	26.93	3.81	29.39	22.49	3.01	24.00	16.97	2.01	1.69	3.51
123	86385	P5	31.18	24.37	3.91	22.45	14.49	1.21	12.41	7.94	-0.79	1.69	3.51
124	86385	GSG	32.62	25.98	4.71	23.92	20.98	2.31	13.49	10.95	0.31	1.69	3.51
125	86389	P3	39.47	28.50	4.41	34.00	19.97	3.81	27.46	21.98	1.01	1.17	3.40

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 M DIL-1	ERYT 4 H DIL-1	INC THICK 4HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H DIL-2	INC THICK 4H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DIL 3	INC THICK 4H DIL-3	TRANS.SUSC .TEST 3	ELISA TEST 3
126	86389	P4	53.48	37.99	6.11	25.98	28.50	7.11	31.98	17.97	2.31	1.17	3.40
127	86389	P5	38.79	24.92	3.91	29.50	19.97	1.81	28.50	19.49	2.81	1.17	3.40
128	86389	GSG	36.41	30.17	6.01	30.94	25.38	3.81	29.00	24.82	2.91	1.17	3.40
129	86511	P3	43.15	24.98	3.51	35.47	24.37	2.81	22.49	20.49	2.31	1.42	2.81
130	86511	P4	47.96	29.93	4.71	38.95	22.91	4.51	31.50	22.49	3.71	1.42	2.81
131	86511	P5	45.89	28.50	6.01	36.78	23.49	2.21	18.49	14.97	1.41	1.42	2.81
132	86511	GSG	40.99	26.93	5.51	38.47	23.37	2.81	24.49	18.55	0.11	1.42	2.81

DATASET : DOSHET-4. Chapter nine. Test 4

CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 M DIL-1	ERYT 4 H DIL-1	INC THICK4 HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H D IL-2	INC THICK4 H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DI L 3	INC THICK 4H DIL-3	TRANS.SUSC EP T.4	ELISA TEST 3
1	8660	P3	36.95	30.98	6.01	29.46	21.45	2.91	20.90	17.00	1.31	0.79	4.91
2	8660	P5	35.87	25.98	4.81	24.00	15.97	2.41	10.95	9.49	0.11	0.79	4.91
3	8660	GSG	32.40	20.35	5.71	25.92	19.44	3.21	17.97	10.00	1.51	0.79	4.91
4	8662	P3	31.61	38.54	3.21	40.50	31.00	3.31	36.22	22.36	1.21	0.86	6.07
5	8662	P5	33.99	25.46	1.51	30.41	21.45	2.51	25.92	17.97	1.11	0.86	6.07
6	8662	GSG	38.42	34.41	4.81	32.40	22.00	3.41	28.98	12.41	0.71	0.86	6.07
7	8664	P3	38.95	24.45	3.51	36.41	22.91	1.11	22.49	19.97	1.41	0.69	3.34
8	8664	P5	36.99	26.93	2.81	26.46	23.00	2.21	11.96	16.49	1.01	0.69	3.34
9	8664	GSG	34.94	22.98	3.51	28.46	21.49	5.01	14.42	11.49	1.51	0.69	3.34
10	8666	P3	37.99	27.50	3.61	34.99	21.00	1.31	29.50	19.97	1.71	0.72	4.65
11	8666	P5	25.92	19.97	1.91	20.00	17.49	3.21	23.49	17.00	2.11	0.72	4.65
12	8666	GSG	29.93	25.92	4.91	34.41	22.00	2.61	21.00	14.49	1.01	0.72	4.65
13	8668	P3	35.33	25.98	3.91	30.94	25.00	2.31	28.14	21.35	0.71	1.15	5.34
14	8668	P5	25.46	28.00	2.81	27.96	25.00	0.81	17.75	14.42	0.31	1.15	5.34
15	8668	GSG	31.94	25.98	4.61	28.50	24.49	2.91	25.51	16.97	0.91	1.15	5.34
16	8670	P3	37.00	16.00	-1.49	30.94	12.49	-2.19	22.00	10.49	0.01	0.68	5.96
17	8670	P5	36.41	12.49	-3.99	31.75	10.95	-1.79	23.00	1.00	-1.99	0.68	5.96
18	8670	GSG	27.93	18.03	1.01	25.50	12.49	-1.29	20.45	11.83	-0.99	0.68	5.96
19	8672	P3	30.74	17.00	3.51	28.93	22.45	2.41	28.28	12.00	0.11	0.72	3.49
20	8672	P5	28.84	23.98	2.61	27.39	19.00	2.01	24.37	20.45	2.61	0.72	3.49
21	8672	GSG	31.94	22.91	6.21	25.98	18.97	3.11	21.21	14.97	2.01	0.72	3.49
22	8674	P3	40.25	33.99	5.31	32.76	24.45	3.61	33.50	23.37	4.11	0.89	4.43
23	8674	P5	29.85	30.94	5.71	30.85	26.98	4.11	12.96	19.97	1.31	0.89	4.43
24	8674	GSG	31.94	29.46	6.01	34.47	29.50	5.61	23.45	22.91	1.71	0.89	4.43
25	8676	P3	41.99	27.98	3.61	39.47	23.98	6.91	21.98	21.49	2.61	0.91	5.96
26	8676	P5	40.47	25.92	2.21	30.94	25.50	2.61	28.39	22.36	-0.09	0.91	5.96
27	8676	GSG	42.95	29.93	4.71	33.00	26.46	3.71	24.45	22.49	2.21	0.91	5.96
28	8680	P3	30.98	25.98	3.71	27.28	18.17	1.51	24.92	17.00	2.01	0.76	4.25
29	8680	P5	27.71	21.98	3.01	21.79	15.97	0.01	16.43	14.42	0.61	0.76	4.25
30	8680	GSG	19.44	15.87	1.01	22.98	15.49	2.81	17.00	10.00	0.51	0.76	4.25
31	8684	P3	43.59	43.86	7.01	32.94	33.50	4.41	29.50	24.37	3.71	0.58	3.69
32	8684	P5	45.69	33.20	4.81	31.46	31.30	5.01	20.49	23.49	1.71	0.58	3.69
33	8684	GSG	35.78	33.99	5.51	28.46	24.98	4.91	22.00	20.98	3.81	0.58	3.69
34	8702	P3	34.47	27.93	7.41	28.93	23.98	4.01	22.49	21.91	3.81	0.97	3.97
35	8702	P5	33.00	34.41	10.71	27.98	25.98	6.81	18.44	20.49	4.51	0.97	3.97
36	8702	GSG	32.00	24.92	6.01	27.50	23.92	8.11	15.00	17.97	2.61	0.97	3.97
37	8708	P3	34.00	25.98	3.31	34.94	23.49	2.31	32.50	19.49	0.61	0.72	5.85

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 M DIL-1	ERYT 4 H DIL-1	INC THICK4 HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H D IL-2	INC THICK4 H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DI L 3	INC THICK 4H DIL-3	TRANS.SUSC EP T.4	ELISA TEST 3
38	8708	P5	28.39	24.45	3.21	25.50	16.97	0.21	23.00	15.00	1.11	0.72	5.85
39	8708	GSG	28.39	23.00	5.01	26.93	20.98	4.91	18.97	21.98	3.01	0.72	5.85
40	8710	P3	43.27	23.49	2.31	30.46	20.98	1.21	28.00	13.49	1.31	0.81	5.06
41	8710	P5	40.40	17.97	2.11	25.38	13.49	1.71	16.97	9.95	0.31	0.81	5.06
42	8710	GSG	24.37	15.00	2.51	21.98	10.00	0.71	19.97	11.00	6.71	0.81	5.06
43	8712	P3	36.66	30.94	3.51	31.40	15.49	2.51	26.00	17.00	1.81	0.88	5.86
44	8712	P5	34.41	18.89	2.61	26.38	17.89	2.71	15.49	11.96	0.11	0.88	5.86
45	8712	GSG	28.50	21.35	4.31	31.50	18.44	3.01	18.97	10.00	0.81	0.88	5.86
46	8716	P3	42.07	18.44	3.11	33.50	12.96	0.51	29.46	13.96	1.71	0.51	5.45
47	8716	P5	26.83	16.31	1.91	23.00	13.86	2.01	14.49	10.00	1.21	0.51	5.45
48	8716	GSG	33.47	19.18	2.01	27.84	14.49	1.91	14.49	8.49	-0.09	0.51	5.45
49	8718	P3	37.50	21.35	2.51	33.50	20.49	2.91	28.84	15.97	2.01	1.08	5.56
50	8718	P5	33.20	27.46	3.21	21.35	20.98	2.91	14.97	17.97	2.21	1.08	5.56
51	8718	GSG	32.40	23.37	4.21	28.39	20.45	3.81	16.97	15.97	2.31	1.08	5.56
52	8722	P3	49.91	16.49	3.41	39.76	12.49	2.21	29.50	9.00	0.61	0.71	5.74
53	8722	P5	34.47	15.00	2.11	31.46	14.49	1.21	24.92	12.96	2.41	0.71	5.74
54	8722	GSG	33.63	12.96	2.31	33.00	9.49	1.11	25.46	1.00	0.31	0.71	5.74
55	85321	P3	39.55	30.46	4.91	42.99	26.46	2.41	36.50	11.96	1.91	1.42	2.34
56	85321	P5	41.95	25.00	3.81	36.33	23.00	1.91	26.83	15.00	1.41	1.42	2.34
57	85321	GSG	31.30	29.50	3.91	31.94	22.98	2.01	18.00	14.42	1.71	1.42	2.34
58	85379	P3	37.95	12.96	2.51	44.96	17.49	2.11	28.39	15.43	0.81	2.01	3.68
59	85379	P5	37.95	20.45	1.91	22.98	16.43	1.21	19.49	11.96	0.91	2.01	3.68
60	85379	GSG	31.00	13.96	2.21	22.91	12.49	1.41	13.00	13.49	0.81	2.01	3.68
61	85381	P3	35.99	32.50	8.11	38.00	33.00	4.91	33.47	23.49	5.21	0.96	3.05
62	85381	P5	29.98	30.46	3.01	27.98	26.46	4.61	20.98	20.00	1.51	0.96	3.05
63	85381	GSG	30.46	32.98	5.61	28.46	26.50	3.81	11.96	20.49	1.81	0.96	3.05
64	85395	P3	30.98	30.94	3.91	26.50	24.49	1.91	17.49	18.44	1.41	1.50	4.16
65	85395	P5	30.94	33.00	4.71	18.49	22.98	2.21	12.49	12.00	0.91	1.50	4.16
66	85395	GSG	29.46	26.27	4.71	23.45	22.00	2.51	13.49	11.00	1.81	1.50	4.16
67	85397	P3	54.39	43.99	9.01	42.00	26.98	4.41	39.50	28.84	4.21	0.83	3.78
68	85397	P5	50.48	39.34	6.61	38.24	30.00	3.51	24.00	27.46	1.81	0.83	3.78
69	85397	GSG	43.99	34.94	5.01	30.46	20.45	2.31	16.31	14.97	2.01	0.83	3.78
70	85405	P3				33.47	30.46	1.31	28.46	20.93	1.31	0.49	4.83
71	85405	P5	38.24	25.00	3.61	29.29	30.46	3.41	16.49	19.00	0.81	0.49	4.83
72	85405	GSG	39.34	31.00	4.91	26.38	24.37	6.21	15.43	17.49	0.51	0.49	4.83
73	86303	P3	31.94	26.38	5.11	23.45	21.00	2.61	1.00	16.73	2.41	0.65	2.36
74	86303	P5	25.46	25.38	4.01	20.35	22.45	2.81	19.44	16.49	1.61	0.65	2.36
75	86303	GSG	28.50	26.50	4.41	24.45	22.98	3.01	14.49	16.43	2.41	0.65	2.36
76	86323	P3	47.48	39.00	9.31	32.86	28.84	8.21	38.00	23.45	3.71	0.81	4.03
77	86323	P5	46.50	39.95	10.91	35.50	30.98	6.01	20.00	25.46	3.51	0.81	4.03
78	86323	GSG	36.50	33.50	7.41	34.94	30.46	11.01	23.98	22.98	4.91	0.81	4.03
79	86333	P3	46.48	26.00	4.01	42.00	22.49	2.71	40.80	22.98	2.61	1.22	3.75
80	86333	P5	38.50	24.92	2.21	36.99	22.98	1.01	24.98	17.32	0.01	1.22	3.75
81	86333	GSG	40.00	26.46	2.71	34.94	24.45	2.61	26.00	12.49	-0.39	1.22	3.75
82	86367	P3	52.99	35.50	7.01	39.69	22.49	4.51	38.95	20.98	3.11	1.24	4.68
83	86367	P5	39.12	23.92	2.51	33.50	27.46	4.21	29.39	22.91	3.31	1.24	4.68
84	86367	GSG	39.42	28.50	4.51	29.50	22.00	3.91	24.98	11.96	3.81	1.24	4.68
85	86369	P3	30.30	15.00	0.81	21.49	10.00	1.31	8.94	10.00	0.61	1.19	4.53
86	86369	P5	31.46	12.96	6.91	22.49	10.95	1.01	11.49	9.49	0.51	1.19	4.53
87	86369	GSG	37.50	18.44	3.81	26.50	12.96	1.41	11.49	8.94	1.01	1.19	4.53
88	86375	P3	35.00	23.45	2.81	24.98	20.49	2.21	16.49	15.97	0.51	1.17	3.72

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CASE No.	ANIMAL	ANTIGEN	OEDEMA 30 MIN DIL-1	ERYT 4 H DIL-1	INC THICK4 HDIL-1	OEDEMA 30 MIN DIL-2	ERYT 4H DIL-2	INC THICK4 H DIL-2	OEDEMA 30 MIN DIL-3	ERYT 4H DI L 3	INC THICK 4H DIL-3	TRANS.SUSC EP T.4	ELISA TEST 3
89	86375	P5	29.00	20.98	2.41	14.42	17.49	1.51	11.49	14.00	1.51	1.17	3.72
90	86375	GSG	33.99	25.92	4.71	30.94	22.98	3.61	20.45	18.00	1.91	1.17	3.72
91	86385	P3	33.00	19.67	2.31	30.46	18.49	2.31	15.97	16.97	1.71	1.21	3.51
92	86385	P5	33.94	25.50	3.61	21.98	18.49	2.11	15.97	15.97	1.01	1.21	3.51
93	86385	GSG	33.47	24.49	3.81	22.98	21.98	3.91	12.41	14.49	2.41	1.21	3.51
94	86389	P3	41.42	31.50	4.51	26.50	31.50	3.21	27.98	21.98	5.71	0.47	3.40
95	86389	P5	30.94	30.50	4.51	24.37	23.98	2.81	13.00	18.57	1.71	0.47	3.40
96	86389	GSG	32.98	22.00	5.41	27.50	20.90	4.91	10.95	11.96	0.11	0.47	3.40
97	86511	P3	44.50	29.39	3.81	36.99	21.45	1.81	19.90	23.45	0.01	0.65	2.81
98	86511	P5	34.50	32.47	5.01	27.93	28.50	3.51	15.49	21.66	1.01	0.65	2.81
99	86511	GSG	35.50	31.98	4.31	28.93	27.98	3.51	16.97	17.49	1.21	0.65	2.81